ORCHESTRATING FEAR RESPONSES IN LARVAL ZEBRAFISH:

A ROLE FOR THE HABENULA

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Abstract

Animals learn to fear stimuli that predict danger, and may flee or freeze in defensive response to those threats. However, pre-exposure to uncontrollable aversive events produce a helpless state that impairs subsequent active avoidance learning, induced by a cascade of stress-induced neural activation in brainstem nuclei. Here, transgenic zebrafish were used to test the involvement of specific habenula neurons in orchestrating active fear responses, as the habenula regulates monoaminergic neurons in the midbrain. In an escapable aversive conditioning paradigm, larval zebrafish learned to avoid a mild electric shock that was predicted by light. KillerRed-mediated optical disruption of habenula afferents caused a deficit in the acquisition of active avoidance, despite the controllable outcome. Instead, larvae switched to freezing-like responses over the course of training, and displayed increased startle. Silencing habenula efferents with expression of the light chain of tetanus toxin similarly altered the conditioned response. These findings identify components of the neural network regulating fear responses in vertebrates, and suggest that the septal-habenula pathway provides a signal for control over a stressor. When disrupted, animals appear unable to downregulate anxiety, and exhibit helpless behavior as if the outcome is uncontrollable. Perturbation of this pathway and consequent dysregulation of monoaminergic systems may contribute to the pathological conditions associated with anxiety disorders.

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Orchestrating Fear Responses in Larval Zebrafish: A Role for the Habenula

What is fear, and why is it vital to physical and mental well-being? Fear is a primal emotion that has evolved to enable animals to deal with danger. It refers to both a psychological state and a system of behavioral and physiological responses that are triggered in reaction to potential threat (Rodrigues, LeDoux & Sapolsky, 2009). When aroused with fear, animals may instinctively display specific action patterns to cope with the threat and escape peril in the environment. Skunks spray foul-smelling musk, hedgehogs roll into a tight ball of spikes, toads puff up their bodies, squirrels head for the nearest tree, and opossums play dead. These defensive mechanisms promote survival of the animal. Humans, too, rely on fear and its relevant responses to save us from jeopardy in various situations. As Rodrigues et al. (2009) put it, "we duck for cover, slam on the brakes, run for the hills, or scream for help" (p. 291).

Further to the expression of defensive behaviors, fear arousal also activates the stress response (LaBar & LeDoux, 2001), an array of transient autonomic and neuroendocrine changes that support the fear reaction. Specifically, monoaminergic systems in the brain release neurotransmitters such as norepinephrine, acetylcholine, serotonin, and dopamine throughout the brain. These neurotransmitters increase arousal and vigilance in the animal and, in general, enhance the processing of external cues (LeDoux, 2007). Blood pressure and heart rate increase, diverting stored energy to muscle and inhibiting digestion. A cascade of hormones is secreted and glucocorticoids circulate through the body and to the brain, further modulating emotional processing (Sapolsky et al., 2000).

Although the stress response facilitates appropriate defensive behaviors, chronic activation may compromise the immune system and contribute to

cardiovascular ailments, as well as pose risk factor for development of pathological states such as specific phobias, generalized anxiety, depression, and post-traumatic stress disorder (Rodrigues et al., 2009). Thus, it is just as important to exit the fear state as it is to enter it, so as to preserve physical and mental well-being. Moreover, extinguishing fear is essential for instrumental learning of avoidance.

While some fear responses are innate, others can be acquired through experience, allowing animals to respond adaptively to circumstances. On encountering an aversive or fearful event, otherwise neutral stimuli presented near or with the event may acquire motivational or emotional value if they are perceived to cue an unpleasant outcome. Subsequent encounters with such stimuli would cause fear arousal and increase the probability of response initiation even when the aversive stimulus has not yet been directly sensed. The fear conditioned stimuli become, essentially, learned predictors of threat or punishment. Then, according to Mowrer's two-factor theory of avoidance (Mowrer, 1951), the desire for *removal* of fear, i.e. obtaining safety, provides a drive-like motivation that can serve as reinforcement for learning and maintaining behaviors instrumental to this end. Thus, fear conditioning and fear reduction are crucial to survival because together they allow organisms to protect themselves effectively in new and changing situations. Not surprisingly, abnormalities in conditioned fear have been evidenced in humans with panic disorders (Lissek et al., 2009), where they exhibit fear in the absence of any real threat.

If we are able to understand the neural mechanisms underlying fear and how they guide the acquisition of avoidance or coping behaviors, we can start to develop effective strategies for treating pathological conditions that arise from dysfunctional fear circuits in the brain. I will begin by providing an overview of research into the neural basis of fear, and outline the significance of dopamine and serotonin

transmission in selecting and depressing appropriate responses, respectively. Next, I will introduce the habenula and explain why it may be critical for defensive behavior through the regulation of both monoaminergic systems. Then, I will describe the experiments, and finally, discuss findings and implications of the present study.

Neural Substrates and Mechanisms of Fear Learning

Studies investigating the neural circuitry of fear mostly focus on learned fear, assessed with fear reactions elicited by a well-defined stimulus (LeDoux, 1995; Maren & Faneslow, 1996). The experimental models often involve a classical conditioning procedure in which the warning (conditioned) stimulus, such as a tone, is contingently paired with an aversive stimulus, such as a mild electric footshock, that instinctively evokes unconditioned circastrike responses like running, jumping, and vocalization. In rodents, the typical behavioral response to such conditioned stimuli is freezing (Faneslow, 1984; Mongeau et al., 2003), which is not elicited directly by the shock but by the fear of its occurrence. Other times, an operant element may be employed wherein the aversive stimulus is omitted if the animal performs a particular behavior. In this case, animals successfully learn to prevent the delivery of shock by making an avoidance response.

The amygdala and periaqueductal gray (PAG) are well-established components of the fear circuitry. Projections from the central nucleus of the amygdala (CEA) to different regions of the PAG have been shown to mediate a range of conditioned fear-related responses, such as freezing via the ventrolateral PAG (De Oca et al., 1998), and bursts of activity (e.g., flight or circastrike) via the dorsolateral PAG (Depaulis, Keay & Bandler, 1992; Faneslow, 1994). Both CEA and PAG project to the nucleus reticularis pontis caudalis, a prominent constituent of the startle circuit,

and modulate fear-potentiated startle (Walker et al, 1997). In addition, projections from CEA to the lateral hypothalamus have been implicated in the control of conditioned cardiovascular responses, and those to the ventral tegmental area and paraventricular hypothalamus modulate vigilance and arousal by conditioned fear (Fendt & Faneslow, 1999). The bed nucleus of the stria terminalis, also connected to the CEA, has been reported to mediate a sustained "anxiety-like" state in contrast to a phasic fear reaction (Duvarci et al., 2009), affecting responses to more diffuse contextual contingencies. The hippocampal formation projects to the amygdala and conveys information about the context of the event, thereby conditioning fear responses to contextual stimuli (Phillips & LeDoux, 1992). These connections are illustrated in Figure 18 in the Appendix.

While lesions of the specific areas can selectively interfere with the expression of individual CRs, damage to the CEA impairs all fear CRs (LeDoux, 2000), suggesting that there are multiple pathways involved in the fear system with the amygdala serving as a key emotive center. When the amygdala detects a dangerous object or situation, it is likely that multiple pathways are activated in concert for different aspects of the fear and stress response, which are presumably fine-tuned by external and internal conditions to shape the appropriate behavioral response (Fendt & Faneslow, 1999). This allows the fear system to be flexible and responsive to variable demands. However, it is not yet clear how the various components are balanced and coordinated into functional behavior.

In principle, an individual animal can respond to a dangerous situation in various ways. For example, rodents may flee or freeze when threatened, depending on the nature of threat and the level of fear it invokes. When conditions appear slightly risky, they become alert. When danger seems imminent – when a predator or warning

cue is sighted – but escapable, they may take flight to avoid attack. When the danger appears inescapable, they may freeze (Blanchard & Blanchard, 1988). Freezing is a prominent defensive strategy because many predators have difficulty detecting an immobile target (Fanselow & Lester, 1988). Mongeau et al. (2003) found that flight and freezing were negatively correlated, suggesting that the responses are in competition with one another, which they postulate to be mediated by opponent neural circuits rather than simple motor incompatibility, because animals that froze in bouts had ample time to display flight behavior but did not. Their behavioral data indicated a shift in the balance of the behaviors from flight to freezing as stress or anxiety increased. Furthermore, a recent study with humans showed that different threat levels invoke activity in different neural systems of the brain (Mobbs et al., 2007). These studies imply the existence of a switch in the neural network that selects for circuits underlying one behavior and inhibits others. This raises the question of how information processed to select the most suitable response for the situation.

To learn the appropriate response, animals probably use internal feedback comparing the actual outcome of an action with the predicted one. Dopaminergic neurons in the midbrain have been implicated in "reward prediction error" signals (Schultz, 1998) that have been proposed to serve this purpose. Specifically, dopamine neurons in the substantia nigra pars compacta show a phasic increase in activity (excited response) if the value of reward is higher than predicted, and a phasic decrease in firing (inhibited response) if the value is lower than expected (Schultz, Dayan & Montague, 1997). Inputs from the dopamine neurons enable the basal ganglia to orient movement based on expected outcome (Hikosaka, Nakamura & Nakahara, 2006). In this way, the dopaminergic system provides a possible mechanism to maximize reward acquisition, that is, to maximize acquisition of

behaviors effective for escaping threat. One important feature of prediction error signaling is that the dopamine neurons stop responding to outcomes on subsequent trials in a contingency block when the outcome becomes predictable by a preceding cue (Schultz, 1998; Matsumoto & Hikosaka, 2007).

Uncontrollable Stress Engenders Maladaptive Fear Learning

If the aversive outcome were inevitable regardless of action, the organism would be unable to organize an appropriate action and may slump into helplessness. Therefore, controllability of the threat is a potent variable determining the animal's behavior towards a stressor. For example, dogs exposed to escapable shock learn to press a panel to terminate the shock, whereas, dogs in a yoked condition receiving equivalent exposure to inescapable shock (because panel pressing did not terminate shock) ceased panel pressing after some trials (Seligman & Maier, 1967). Interestingly, the dogs in the inescapable shock group subsequently failed to jump a barrier to prevent shock delivery during avoidance training, even though this entailed continued exposure to the painful stimulus. Dogs with prior exposure to escapable shock did not differ from untreated dogs in avoidance training 24 hours later; they successfully jumped the barrier. Only those with no control over the stress experience later showed avoidance deficits, as well as exaggerated fear conditioning (Osborne et al., 1975) and increased anxiety (Short & Maier, 1993). This effect has been demonstrated in a range of species, including rats (Maier, 1990) and humans (Thornton & Jacobs, 1971), and has been termed "learned helplessness".

In an uncontrollable situation, prediction error shaping of responses would be deemed ineffective and other transmitter systems may dominate. As expectations of the learned negative outcome actualize, reward prediction errors would no longer

contribute to resulting behavior. Instead, a different monoaminergic system comes into play. Inescapable shock activates serotonin (5-HT) neurons in the dorsal raphe nucleus (DRN) significantly more than does equal amounts of escapable shock (Grahn et al., 1999), resulting in greater extracellular 5-HT within the DRN and in projection regions such as the amygdala (Amat et al., 1998) and the medial prefrontal cortex (Bland et al., 2003). 5-HT efflux within the DRN sensitizes the neurons by desensitizing inhibitory 5-HT1_A receptors to produce exaggerated release of 5-HT in projection regions upon subsequent footshocks (Maier & Watkins, 2005). This activation is necessary to produce the behavioral effects of uncontrollable stress, as infusion of the 5-HT1_A agonist 8-OH-DPAT (Maier, Grahn & Watkins, 1995) or lesion of the DRN (Maier et al., 1993) block learned helplessness. Moreover, stimulating 5-HT neurons in the DRN inhibits flight behavior via projections to the dorsal PAG, and potentiates fear and anxiety via projections to the amygdala (Maier & Watkins, 2005).

On subsequent transition to a controllable situation, it is possible that the individual carries over a state of sensitized serotonin and possibly overshadowed dopamine activity, which result in helpless behavior despite avoidable outcomes. The impression of helplessness is self-fulfilling, since lack of a coping response subjects the individual to consistent negative experience only to be further expected. Based on this speculation, changes in the balance of monoaminergic systems produce varying responses to threat.

Regulating Monoaminergic Systems: Connections to and from the Habenula

Having discussed the importance of the dopaminergic and serotonergic systems in the neural circuits that underlie fear conditioning, there is good reason to

turn attention to the habenula, an epithalamic brain structure that regulates a range of midbrain targets, including dopaminergic neurons in the substantia nigra pars compacta (Christoph, Leonzio & Wilcox, 1986; Ji & Shepard, 2007) and serotonergic neurons in the raphe nuclei (Wang & Aghajanian, 1977; Yang et al., 2008). In fact, the habenula is one of few brain regions that influence both dopamine and serotonin systems (Hikosaka, 2010).

Sutherland (1982) described the habenular complex as a major component of the dorsal diencephalic conduction pathway connecting the limbic forebrain and the midbrain. Anatomically, the habenula consists of a commissure and two distinct nuclei in each hemisphere, termed the medial and lateral habenula in mammals. The majority of afferent fibers travel to the habenula in the stria medullaris and efferent fibers travel away from the habenula in the fasciculus retroflexus. The medial habenula receives its main source of input from the posterior septal area, primarily from the nucleus fimbrialis septi and the nucleus triangularis septi, with minor contributions from the ventral PAG, the nucleus of the diagonal band of Broca and the nucleus accumbens. The lateral habenula receives converging input from the entopeduncular nucleus (non-primate homolog of the globus pallidus internae), lateral preoptic and lateral hypothalamic areas, with only few afferents from the septum, namely, the lateral septal nucleus. The nucleus of the diagonal band of Broca and the nucleus accumbens also supply minor inputs. These areas appear to be the only forebrain regions that project to both medial and lateral habenula nuclei. The lateral habenula also receives descending projections from the medial frontal cortex and the bed nucleus of the stria terminalis (Lecourtier & Kelly, 2007). These connections are illustrated in Figure 18 in the Appendix.

Notably, there is additional evidence of ascending noradrenergic fibers to the medial and lateral habenula from the ventral PAG, as well as serotonergic innervations to medial and lateral habenula from the median raphe, and dopaminergic innervations to the lateral habenula from the ventral tegmental area of Tsai (Sutherland, 1982). The monoaminergic signals may serve as feedback mechanisms providing information about the outcome to guide ongoing behavior.

In a series of electrophysiological studies with primates, Matsumoto and Hikosaka (2007; 2009) reported that the lateral habenula neurons increased activity in response to cues predicting delivery of aversive stimuli, or omission of appetitive stimuli, which in turn inhibited dopamine neurons in the substantia nigra pars compacta. Hence, they proposed that the lateral habenula preferentially represents unpleasant events across distinct contexts, and is involved in motivational control of behavior through modulation of the reward response of dopamine neurons. It is not known whether aversive stimuli induce changes in activity of medial habenula neurons, perhaps because its inaccessibility in mammals makes it difficult to perform electrophysiological recordings. However, some rodent studies report stress-induced immunological responses in the medial habenula, such as increased levels of pro-inflammatory cytokine IL-18 (Sugama et al, 2002) and increased numbers of mast cells (Cirulli et al, 1998).

Lesioning the Habenula: Effects on Fear Conditioning

Given this pattern of connectivity and activity, the habenula may play a pivotal role in the learning and orchestration of defensive behaviors. Indeed, lesion studies with rats have provided some evidence of this, although consequences of habenula damage appear discrepant. On the one hand, electrolytic and radio-

frequency lesions of the habenula produced deficits in active avoidance learning (Thornton & Bradbury, 1989; Thornton et al., 1994; Wilcox et al., 1986). Specifically, the rats displayed a tendency to freeze in response to the conditioned stimuli instead of executing avoidance behavior, but demonstrated no difficulty in reacting to shock with proper motor responses. This implies that the lesions did not remove sensitivity to shock or impair motor abilities. The rats appeared to have acquired a conditioned emotional response, albeit ineffectually. On the other hand, habenula lesions eliminated the avoidance deficits that normally follow exposure to an uncontrollable stressor (Amat et al., 2001). At the level of neurotransmission, the habenula lesions attenuated the rise of extracellular serotonin levels in the DRN otherwise observed in sham-operated controls exposed to inescapable shock. Thus, in general, the habenula appears to be necessary for the modification of monoamine transmission and behavioral responses during encounters with aversive and stressful events.

The varied behavioral results may be due to lesions (a) damaging variable regions within or beyond that intended; (b) destroying fibers of passage through the habenula; (c) extending to different subregions of the habenula involved in separate functions. These considerations are not trivial, given findings that (a) the rat with the greatest rostral habenula sparing of all the habenular-lesioned rats in Thornton et al.'s (1994) study displayed the most evidence of avoidance learning; (b) a significant number of fibers in the stria medullaris pass through the habenula, without terminating, as they project to the midbrain tegmentum from the septum (Sutherland, 1982); (c) immobilization stress induced activation within the medial, but not the lateral, portion of the lateral habenula (Wirtshafter, Asin & Pitzer, 1994), indicating distinct neural pathways and possibly functional differentiation of these two regions.

Interestingly, Wilcox et al. (1986) additionally employed a different lesion technique using injections of kainic acid, a cytotoxin shown to selectively destroy cell bodies while sparing fibers of passage through the structure, but did not find any avoidance deficits. In this experiment, degeneration was neither observed in the medial habenula nor its fibers projecting through the core of the fasciculus retroflexus bundle to the interpeduncular nucleus, consistent with previous findings that the medial habenula is insensitive to cytotoxic effects of kainic acid. In contrast, the neuronal cell bodies in the lateral habenula and their fibers surrounding the core of the fasciculus retroflexus showed extensive degeneration indicating substantial damage. Thus, it was suggested that the impaired avoidance performance arises from disruption of the septal-medial habenula-interpeduncular nucleus pathway. Evidence that lesions of the septal nuclei (Ross & Grossman, 1977), the interpeduncular nucleus (Thompson, 1960), and transections of the stria medullaris (Ross, Grossman & Grossman, 1975) impair active avoidance responding supports this hypothesis.

Investigating the Role of the Habenula in Zebrafish

It is undeniable that lesion studies need to be definite about the brain tissue subject to manipulation, in order to accurately assess and interpret the effects of damaging the neural substrate of interest. Components of networks in the brain that mediate behavior are neurons rather than discrete brain regions. Therefore, manipulating specific sets of neurons offers a more precise method of investigating the circuits that underlie fear responses, especially when the substrate of interest is a node in the network, such as the habenula.

To achieve this, we developed a learned avoidance assay in larval zebrafish. These young animals are well suited for precise disruption of neural circuits through

the use of tractable transgenic techniques, which target expression of foreign proteins in subsets of neurons. In addition, they have a prominent habenula, are translucent, and exhibit a range of complex behaviors from early life stages (Baier & Scott, 2009). The fundamental premise is that the fear circuitry in zebrafish is comparable to that in mammals, conserved across evolution. Ray-finned fishes (*Actinopterygii,* to which belong *Teleostei*, and in turn, *Danio rerio*) and land vertebrates (*Tetrapoda*) share a common ancestor dating back some 400 million years ago, from which both have inherited similar features of brain organization (Braford, 1995). The fear system serves evolutionarily useful function selected for across generations; as a module of ancient origin, the neural circuits are likely situated in subcortical and brainstem regions that comprise primitive brains before taxa with more developed cortices emerged (Öhman & Mineka, 2001). This is in consonance with the substrates of fear presently identified in mammals and in line with the fact that the fear system is activated automatically in every species, that is, independent of consciousness and relatively immune to cognitive influences (Öhman & Mineka, 2001). Several homologs of the neural substrates have also been defined in zebrafish (see Jesuthasan, 2011). Moreover, innate fear in the zebrafish manifest as flight and freezing behavior (Jesuthasan & Mathuru, 2008), similar to that observed with rodents. Thus, the specific components and circuits underlying fear are essentially retained and can be relevantly studied over the range of animal species, including zebrafish.

The present series of experiments investigated the role of the habenula in fear learning and control of behavior in response to aversive stimuli. We employed two different methods of disrupting the neural circuits involving the habenula in larval zebrafish, and tested the animals in a fear-learning paradigm. Experiment 1 describes the learning paradigm and variety of behaviors exhibited to the conditioned stimulus,

depending on the nature of the outcome. In Experiment 2, an optogenetic tool involving a photosensitizer, KillerRed, was used to damage afferent neurons in a spatially and temporally controlled manner, while in Experiment 3 a genetically encoded protein, tetanus toxin light chain, was used to silence specific efferent neurons of the habenula.

Experiment 1

This experiment investigated zebrafish behavior in response to a light stimulus following exposure to a fear-conditioning paradigm developed for larval fish. It provides empirical evidence of learned fear responses in the fish, which varied depending on the circumstance encountered in the different conditions. All fish had a normal habenula in both hemispheres.

Method

Animals. Zebrafish (*Danio rerio*) were maintained in groups of 20 at 28°C, fed twice a day with spawn powder and live baby brine shrimp until immediately prior to the experiments. Animals of approximately seven to eight mm in length (20-40 days post fertilization) were randomly assigned to groups (n=10) and tested during the light portion of the fish's light-dark cycle, within the 0800-2000 hours time window, and in accordance with the Animal Care Policy of Neuroscience Research Partnership–Institutional Animal Care and Use Committee.

Fear conditioning. The fear-learning paradigm was conducted in a shuttle box (Figure 1) comprising a clear tank (35 x 80 x 30 mm) filled with 50 ml of embryo water (NaCl, KCl, CaCl2, and MgSO4 dissolved in solution; 840 μSm^{-1}), giving a water level of 180 mm throughout the tank. Each long side of the tank was lined with

two (30 x 30 mm) stainless steel electrode plates to deliver a mild electric shock as the aversive unconditioned stimulus (US; 0.86V/mm, single pulse, 100 msec) on either side of the tank, thus virtually dividing the shuttle-box into two chambers of equal size. The tank was placed in a black test box, with a red LED mounted in the test box wall at each end of the tank as the conditioned stimulus (CS; five sec). The LED and stimulator (Grass Technologies SD9) were computer controlled using E-prime 1.1 SP3 software (Psychology Software Tools, USA).

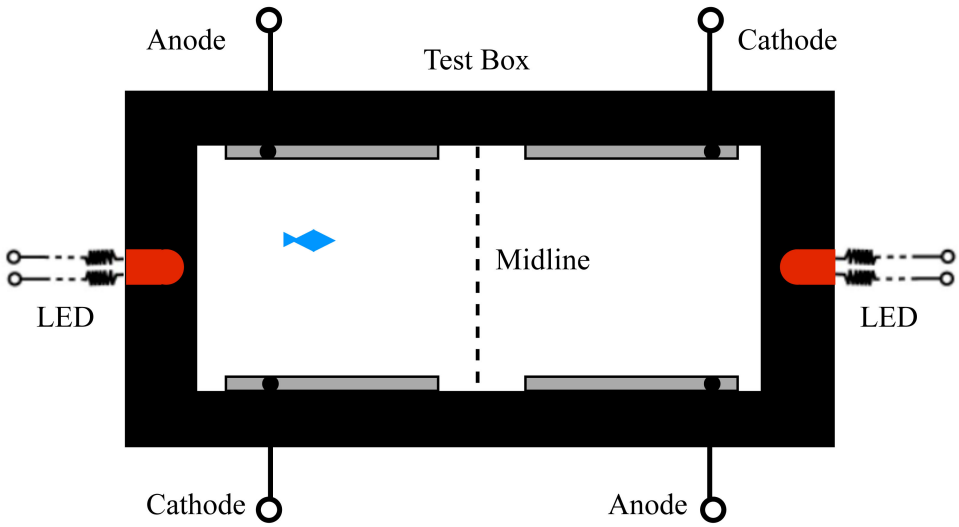

Figure 1. Schematic representation (top view) of the shuttle box apparatus used for fear conditioning. The larval zebrafish illustrated in blue is presented to scale.

Larval zebrafish were trained and tested individually. To begin, the fish was introduced into the middle of the shuttle box and given a 15-minute habituation period before commencing training. This time window allowed any erratic movement to decrease to stable swimming pattern (Lee, 2008), presumably minimizing any extraneous fear of the novel environment. Fish trained on the escapable paired (ESP) procedure received 10 presentations of the five second CS, co-terminating with the

100 millisecond US. For each trial, the CS and US were both presented on one side of the shuttle box only, with side determined by fish position at the scheduled time of CS presentation. The inter-trial interval (ITI) varied between 4.5 – 5.5 minutes, with an average duration of five minutes. In an explicitly unpaired control procedure, the light was presented 10 times, but never co-terminated with shock. Instead, 10 separate shocks were delivered pseudo-randomly within the ITIs, always to the side of the tank where the fish was located. Additionally, a CS alone control was conducted wherein the light was presented for 10 trials, but no shock was delivered during the session. In all conditions, Trial 11 was a probe trial in which fish were exposed to five seconds of light alone in the absence of shock.

To alter the nature of the threat, another group of fish were trained on an inescapable paired (ISP) procedure, receiving the same presentations of CS on one side of the shuttle box, but with the US delivered to both sides of the tank instead of one. Comparing the setups, the aversive outcome is considered "escapable" in the ESP because an electric field applied to only one side of the tank diminishes with increasing distance from that side of the tank, hence making the outcome less unpleasant; whereas, the unpleasant outcome is "inescapable" in the ISP since the electric field is equally present on both sides of the tank.

To examine the effects of uncontrollable stress on subsequent behavior in avoidance learning, a separate group of fish were first subjected to an inescapable shock (IS) treatment, then immediately transferred to the shuttle box for ESP training.

Pre-exposure to inescapable shock (IS). Ten fish individually received pre-training exposure to inescapable shock in a separate clear tank (45 x 70 x 25 mm) filled with 50 ml of embryo water (840 μSm^{-1}), and placed in a white test box. Each

long side of the tank was lined with a stainless steel electrode plate (60 x 30 mm) to deliver electric shock throughout the tank. Each fish was placed in the tank for a five-minute habituation period before commencing 10 trials of inescapable shock with an average ITI of two minutes. On each trial, a pulse train of 100 millisecond shocks (0.86V/mm, two pulses/sec) was delivered for a period of five seconds.

Behavioral analyses. Fish behavior was video recorded (25 frames/sec) using an Apple i-Sight camera and analyzed with ImageJ 1.39u software (National Institutes of Health, USA). For each trial, 15 seconds of the video recordings (five sec pre-CS, five sec during CS, and five sec post-CS) were analyzed for the position of the fish in its swim path; in particular, when in time the fish crossed the virtual midline of the tank into the opposite side. Each fish was coded for whether or not it crossed over to the other side of the tank during the five second CS presentation.

The 15-second videos were also analyzed for swim speed. The swim path was traced, and then time and distance plotted in a kymograph. Next, gradients of the kymograph were calculated, and speeds obtained in one second bins. Startle responses, defined as a minimum two-fold increase in swimming speed from baseline within the first second after CS onset, were coded as present or absent for each fish.

Statistical analyses. To evaluate differences in the proportion of fish crossing the midline during the CS, as well as the proportion of fish displaying a startle response, two-way Chi-square tests were performed across the conditions of interest.

In analyzing swim responses to the CS, we compared mean swimming speed during the fifth second after CS onset (that is, the one second preceding CS offset) in the probe trials across training conditions, controlling for baseline speed during the one second preceding CS onset. This time window was selected as the unit of analysis

for two reasons. Based on earlier work in developing the assay, the final second included the most distinct behavioral changes to the CS, relative to the baseline activity of the fish, to compare across training conditions. Also, the final second of the CS reflects behavior of the fish as the expected time of shock approaches in the paired (ESP and ISP) conditions. Thus, it most suitably indicates responses to the CS that result from learning. One-way analyses of covariance (ANCOVA) were conducted on the data, using the baseline speed as the covariate. To ensure that the assumptions of ANCOVA were met, models were generated before each analysis to confirm that the regression slopes relating the covariate to the dependent variable were equal across groups. In other words, differences on the CS speed among groups did not vary as a function of baseline speed. In all our analyses, the Group X Baseline Speed (i.e., the covariate) interaction was not significant, indicating homogeneity of slopes. To test for normality of the data, histograms of standardized residuals were generated and examined for a normal distributional shape.

For all statistical analyses, when follow-up pairwise comparisons were required, Holm's Sequential Bonferroni Method was used to control for Type I error at the 0.05 alpha level. Where applicable, the adjusted α_{pc} is indicated in parentheses.

Results and Discussion

The fish were assessed for whether they made a response to move away from the illuminated LED and cross the virtual midline of the tank within the five-second presentation of the CS (light). Since the electric shock was applied to only one side of the tank during escapable shock (ESP) training, the intensity of the electric field diminished as the fish moved further away from the locus of the threat. Therefore, such a response was interpreted as avoiding the brunt of the shock, making the

experience less aversive. Comparing the paired ESP group with the explicitly Unpaired and CS-alone controls, only fish that experienced CS-US pairings displayed the crossover response in the probe trial (Figure 2A). A two-way contingency table analysis indicated significant group differences (Pearson χ^2 (2, N=30) = 18.095; p < .001; Cramer's V = .777), and follow-up pairwise comparisons found a significantly higher level of avoidance response in the ESP condition compared to the Unpaired condition (χ^2 = 13.333; p < .001 (α_{pc} = .017)) and the CS-alone condition (χ^2 = 9.899; p = .002 (α_{pc} = .025)).

Midline crossing was accompanied by an increase in swimming speed, mainly during the final second of CS presentation (Figure 2B). An ANCOVA controlling for pre-CS speed indicated a significant group effect (F (2, 26) = 9.035; p = .001; partial η^2 = .41), and pairwise comparisons showed statistical differences between the ESP group and the Unpaired group (p = .001 (α_{pc} = .017)) as well as the CS-alone group (p = .002 (α_{pc} = .025)). There were no significant differences between the control groups in avoidance (χ^2 = 1.053; p = .305 (α_{pc} = .05)) or speed (p = .589 (α_{pc} = .05)).

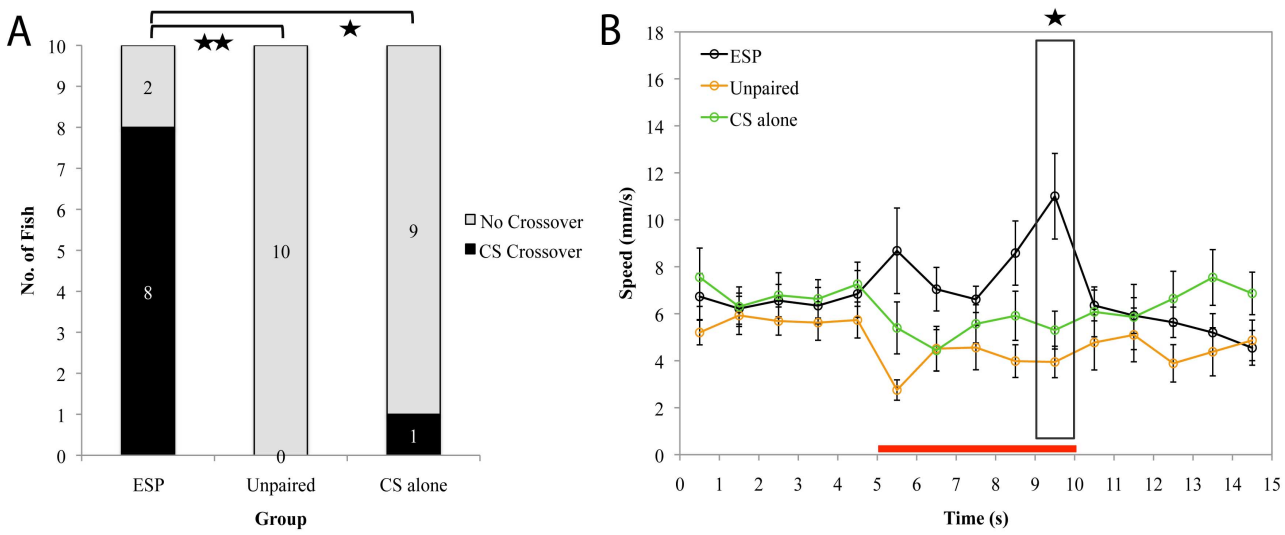

Figure 2. Midline crossover performance (A) and swimming speed (B) of the ESP, Unpaired, and CS alone groups in the probe trial. The red bar indicates CS presentation, and the box indicates the time point during CS presentation for which the ANCOVA analysis was conducted on swimming speeds between groups, using pre-CS speed as the covariate. Error bars indicate s.e.m., ★★ $p < .001$; ★ $p < .05$.

When the electric shock was applied to both sides of the tank during inescapable shock (ISP) training, the paired ISP fish displayed a different conditioned response in the probe trial (Figure 3) as compared to the ESP fish. Unlike ESP fish, significantly fewer ISP fish crossed the midline away from the LED (Pearson χ^2 (1, N=20) = 9.899; p = .002; Cramer's Φ = .704); instead, there was a burst in swimming speed immediately after light onset, followed by reduced mobility until light offset (F (1, 17) = 16.146; p = .001; partial η^2 = .487). In other words, the larval zebrafish responded differently to the CS when the aversive outcome during training was escapable versus inescapable.

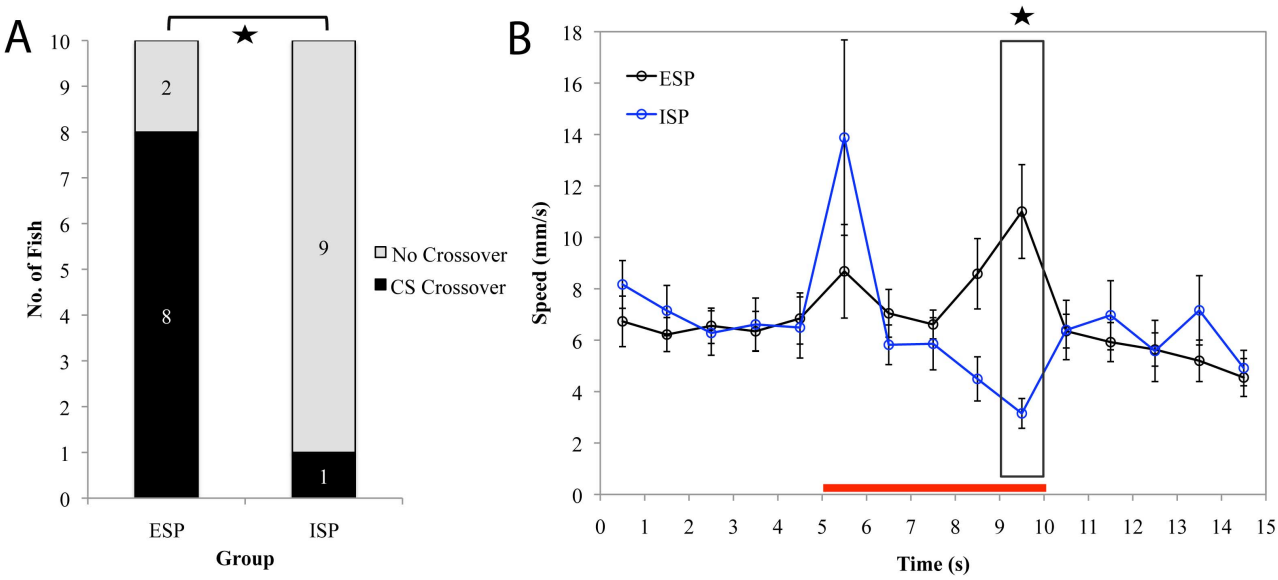

Figure 3. Midline crossover performance (A) and swimming speed (B) of the ESP and ISP groups in the probe trial. The red bar indicates CS presentation, and the box indicates the time point during CS presentation for which the ANCOVA analysis was conducted on swimming speeds between groups, using pre-CS speed as the covariate. Error bars indicate s.e.m., ★ $p < .05$.

The experience of inescapable shock not only changed the conditioned response in ISP training, but also altered avoidance learning during subsequent ESP training (Figure 4). When pre-exposed to inescapable shock before escapable shock conditioning (IS→ESP), the fish did not exhibit avoidance responses (midline crossovers) in the probe trial (Pearson χ^2 (1, N=20) = 13.333; $p < .001$; Cramer's ϕ = .816). In contrast to ESP fish without pre-exposure to inescapable shock, IS→ESP fish slowed down until CS offset (F (1, 17) = 14.156; $p = .002$; partial $\eta^2 = .454$). The swimming trajectories presented in Figure 5 clearly illustrate the difference in behaviors across the conditions.

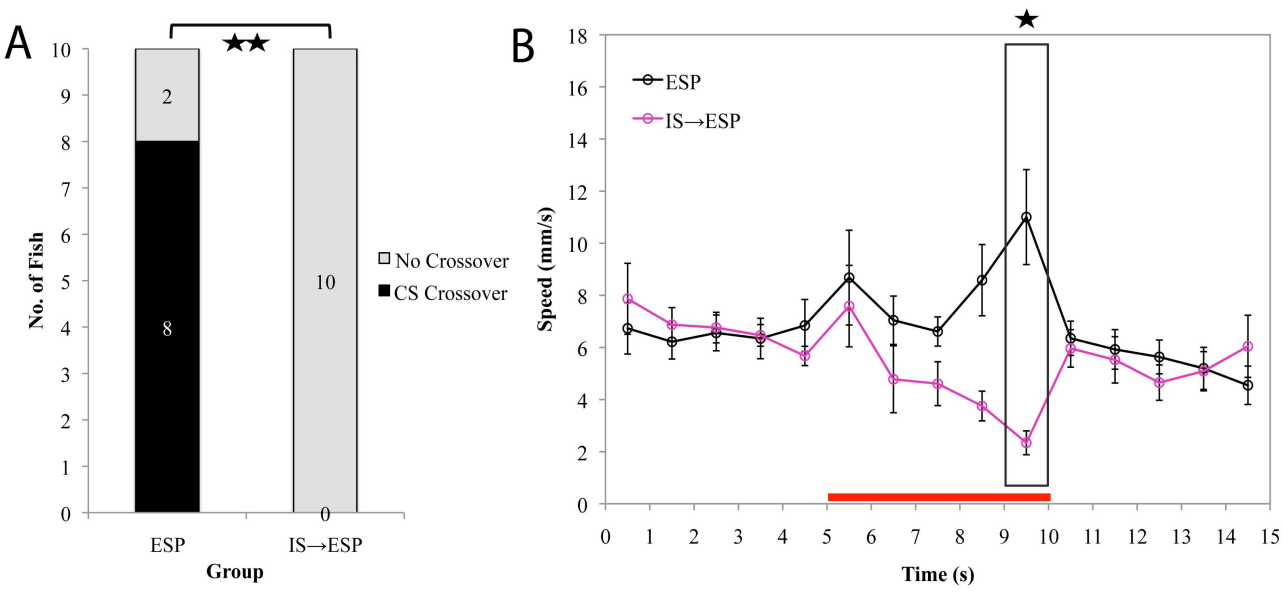

Figure 4. Midline crossover performance (A) and swimming speed (B) of the ESP and IS→ESP groups in the probe trial. The red bar indicates CS presentation, and the box indicates the time point during CS presentation for which the ANCOVA analysis was conducted on swimming speeds between groups, using pre-CS speed as the covariate. Error bars indicate s.e.m., ★★ $p < .001$; ★ $p < .05$.

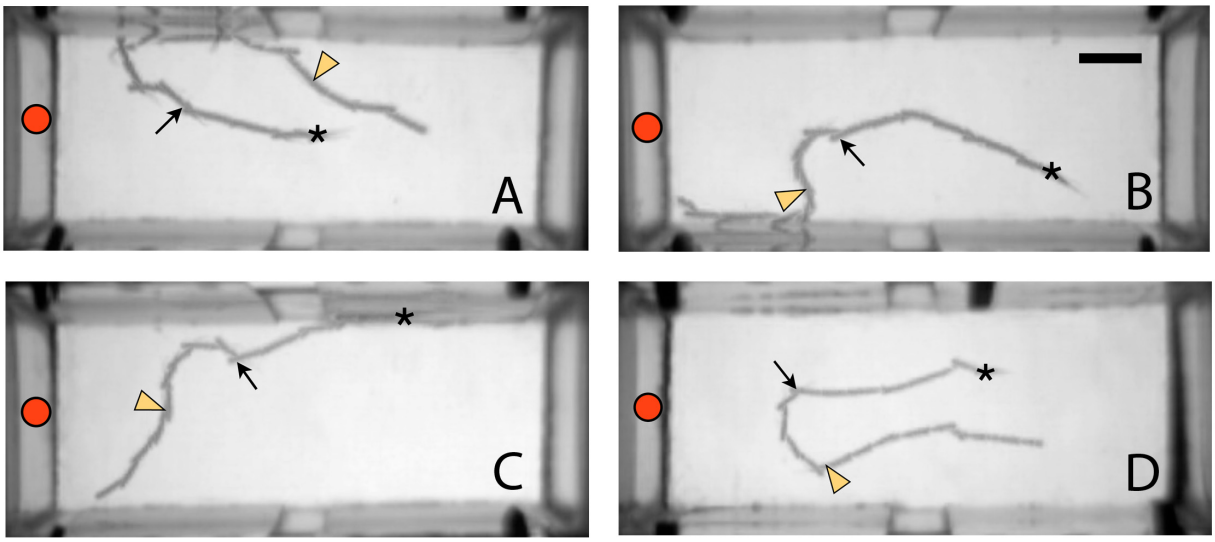

Figure 5. Swimming trajectories of a representative individual fish in the probe trial for each of the four conditions, 5 seconds before, during, and after CS presentation. A: ESP condition; B: Unpaired condition; C: CS alone condition; D: IS→ESP condition. The black asterisk indicates fish location at the start of the 15 seconds. The black arrow indicates fish location at CS onset, while the yellow arrowhead indicates fish location at CS offset. The red circle indicates the position of the LED. Scale bar = 1 cm at midlevel of chamber.

Of note, fish displayed initial avoidance responses early in conditioning, but these diminished across training trials in the inescapable shock (ISP and IS→ESP) conditions, while increasing over the training session in the ESP group (Figure 6). The Unpaired and CS-alone control groups did not display an increase in avoidance responding at any time during the session.

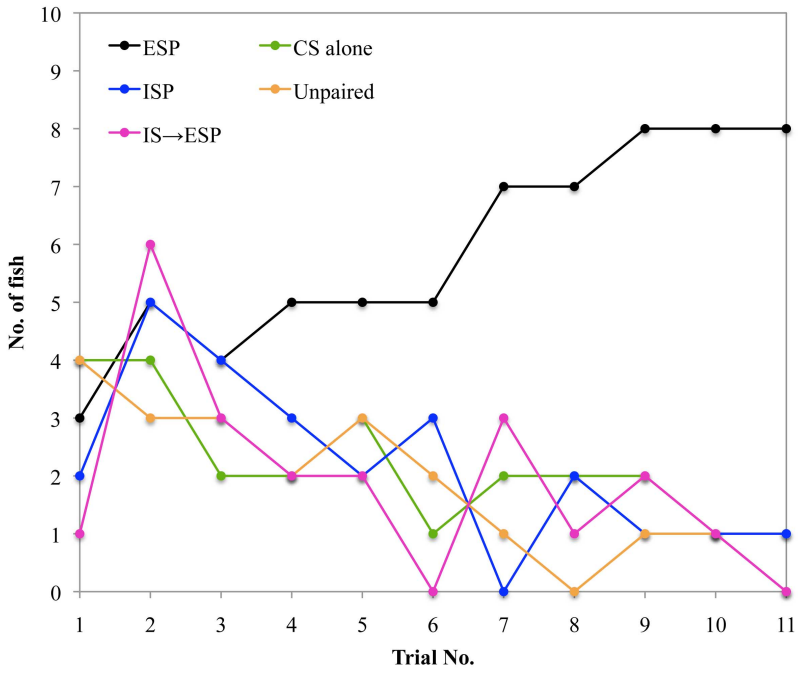

Figure 6. Number of fish, out of 10, that crossed the midline during CS presentation for each of the 10 training trials and the probe trial (Trial 11, shock not presented).

Experiment 2

In this experiment, learned fear responses were tested after optical disruption of the neural pathway supplying input to the habenula. These neurons expressed a genetically encoded photosensitizer, KillerRed, which mediated the manipulation. KillerRed is a red fluorescent protein that rapidly bleaches and generates reactive oxygen species (ROS) upon excitation with green light (540-580 nm). When targeted to the membrane, light-induced production of ROS presumably results in oxidation of

lipids at the membrane, thus perturbing its activity. Indeed, Bulina et al. (2006b) demonstrated cell fragmentation and death within the 30 minutes following 10-minute green light irradiation of human HeLa cells in culture. Since zebrafish larvae are sufficiently translucent to allow light penetration *in vivo,* the optogenetic approach enables light-driven spatial and temporal control over the intact nervous system.

Method

Generation of transgenic zebrafish lines. KillerRed-expressing enhancer trap lines were generated using the membrane-tethered version of KillerRed containing the Neuromodulin membrane localization signal sequence (http://www.evrogen.com/products/vectors/pKillerRed-membrane/pKillerRed-membrane.shtml). The orginal Tol2 transposon pBK-CMV enhancer trap plasmid (Tol2-GFP) was modified to contain the partial *krt4* promoter driving expression of KillerRed (Parinov et al., 2004). Briefly, the GFP reporter flanked by 5' BamH1 and 3' Not1 was replaced by the KillerRed flanked by the same sites. The Tol2-KillerRed plasmid was co-injected with transposase mRNA into one to four cell stage zebrafish embryos. Carriers expressing the KillerRed transgene with tissue-specific expression patterns were maintained and outcrossed with AB wildtype fish upon reaching sexual maturity. Offspring expressing KillerRed were then raised to adulthood, generating F_1 of the enhancer trap lines expressing membrane-targeted KillerRed. Of these, two specific transgenic lines were used in the present experiment, both kindly provided by Vladimir Korzh. In one fish line, named KR11, KillerRed is expressed in habenula afferents from the ventro-lateral forebrain. In the second line, KR4, KillerRed is expressed in cells of the circumventricular organ and the parapineal organ, situated close to the habenula.

Photobleaching of KillerRed-expressing cells. KR11 and KR4 fish were temporarily anesthetized with MS 222 (methyl3-aminobenzoate methanesulfonate; Sigma) dissolved in embryo water, mounted dorsal-up in 1.2% low-melting agarose (in embryo water), immersed in fresh embryo water, and viewed with a 20x water immersion objective on a Leica DM LFS microscope. Using a mercury lamp (100w) and TRITC filter (515-560 nm excitation), habenula afferent neurons were irradiated for 40-60 minutes until the KillerRed fluorescence was not detectable. The region of illumination was minimized using the field diaphragm, to maximize illumination intensity. The embryo medium was bubbled continuously with oxygen throughout the procedure, as oxygen partial pressure is known to affect the efficiency of oxygen-based ROS generation (Bulina et al., 2006a).

Fear conditioning (ESP) was carried out after a three-hour rest period, allowing time for cell damage and for the fish to recover from the procedure. An additional KR11 group was first trained on the ESP, before undergoing irradiation. Thereafter, they were kept in a holding tank for a three-hour interval, then re-introduced to the conditioning apparatus and administered the probe trial. This sequence of procedures was aimed at dissociating acquisition and performance deficits caused by the photodisruption. If photobleaching tampered with acquisition mechanisms, irradiation after training trials would not affect the animal's ability to learn and execute the avoidance response in the probe trial. However, if photobleaching perturbed performance mechanisms, irradiation after training trials would still impact behavior on the probe trial, as the disruption would interfere with execution of the response.

Unpaired, CS alone, and US alone control procedures were also conducted with separate groups of irradiated KR11 fish, three hours after irradiation was completed.

Annexin V labeling. To determine degree of damage to the cell after photobleaching of KillerRed-expressing neurons, the left habenula was photobleached in a separate procedure while the right habenula was left intact as an internal-subject control. Three hours later, Fluorescein isothiocyanate (FITC)-conjugated Annexin V (50 μg/ml; Sigma) was injected into the forebrain using an air pressure injector (FemtoJet; Eppendorf). Annexin V binds to malondialdehyde (MDA), a major product of lipid peroxidation, which introduces negative charges that affect the interfacial ionic layer of the cell membrane (Balasubramanian et al., 2001). Thus, positive Annexin V labeling indicates lipid peroxidation, the reaction of polyunsaturated fatty acids with active oxygen that disrupts the integrity of cell membranes and impairs action potential generation (Pellmar & Lepinski, 1992; Pellmar, 1986). Conjugation with the FITC fluorophore enables injection and expression of the label to be monitored using green fluorescence detected under the microscope. 10 minutes after the injection, fish were imaged every half hour for three hours, using confocal microscopy.

In a separate procedure to track the rate of labeling, FITC-conjugated Annexin V was microinjected into the forebrain, and followed by 40 minutes of irradiation, photobleaching KillerRed in both the left and right habenula. Images were taken every two minutes for the first 10 minutes, and then every 10 minutes for 40 minutes.

Immunofluorescence. In an effort to characterize the neurons expressing KillerRed, antibody labeling of chemical markers was performed. Protein-

immunoreactivity enables different functional subpopulations of cells to be distinguished, and can be used to identify specific neuronal populations in the central nervous system. Such analyses may help to elucidate homologies across species of animals and facilitate comparative understanding of the neural substrates of interest.

Brains of 30 days-post-fertilization (dpf) fish were dissected out and fixed overnight at 4°C with 4% paraformaldehyde (PFA) prepared in phosphate buffered saline (PBS). A solution of PBS with 1% bovine serum albumin (Fraction V; Sigma), 1% DMSO and 0.1% Triton X-100 was used to permeabilize the tissue and to dilute primary antibodies. Brains were washed three times in the solution with half hour intervals, and then incubated overnight in the primary antibody for at least 12 hours at 4°C. After which, they were rinsed three times with half hour intervals in PBS and then incubated in the secondary antibody for two hours at room temperature. PBS was used to dilute secondary antibodies. Finally, after three further rinses, the brains were stored in PBS at 4°C until they were mounted in 1.2% low-melting agarose (in PBS), and imaged with a laser scanning confocal microscope (Zeiss LSM 510), using 20x, 40x and 63x water immersion objectives.

The primary antibodies used were calretinin (Swant 7699/4; 1:2000 dilution), GABA (Chemicon AB131, 1:500), and VGlut1/2 (Synaptic Systems 135503; 1:100), which recognize target proteins within cells. The secondary antibodies used were Alexa 488 goat anti-rabbit (Molecular Probes; 1:500) and Alexa488 goat anti-mouse (Molecular Probes; 1:500), which carry the Alexa 488 fluorophore and bind to the primary antibodies, enabling detection with fluorescence microscopy.

Results and Discussion

KillerRed expression. The dorsal habenula in zebrafish is homologous to the mammalian medial habenula, while the ventral habenula is homologous to the mammalian lateral habenula (Amo et al., 2010). In the KR11 fish, KillerRed was expressed in the membrane of neurons innervating the dorsal and ventral habenula from the ventro-lateral forebrain (Figure 7A-D). This cluster is the largest source of input neurons to the habenula in teleost fish (Hendricks & Jesuthasan, 2007; Yañez & Anadón, 1996) and may include the bed nucleus of the stria medullaris (BNSM), derived from the eminentia thalami (Mueller & Guo, 2009). In adult zebrafish, Mueller and Guo (2009) identified the BNSM as a GAD67-negative nucleus that surrounds the lateral forebrain bundle (lfb in Figure 7G) at anterior levels, and appears as a solid nucleus dorsal of the lateral forebrain bundle at more caudal levels. In rodents, the BNSM is a caudal extension of the septal region (Risold & Swanson, 1995), where neurons are calretinin-positive (Abbott & Jacobowitz, 1999) and project fibers to discrete subnuclei in the medial habenula via the stria medullaris (Shinoda & Tohyama, 1987). Antibody labels in KR11 fish indicated calretinin expression overlapping with a subset of KillerRed-expressing neurons (Figure 7E), suggesting that the cluster of afferents includes the bed nucleus of the stria medullaris (BNSM). Interestingly, a cluster of calretinin-positive neuronal cell bodies were seen in the medial subnucleus of the dorsal habenula, in line with Shinoda and Tohyama's (1987) report that the BNSM projects to the medial habenula in rodents. Being the posterior-most part of the septal area, it is likely that the BNSM is a migration of neurons, related to the other septal nuclei by embryonic origin.

The major septal nuclei that innervate the mammalian medial habenula – namely, the nucleus septofimbrialis (SFi) and the nucleus triangularis (TS) in the

posterior septal area – express VGlut2, a marker for glutamatergic synapses, but not GAD67, a marker for GABAergic neurons (Qin & Luo, 2009). A similar pattern was detected in KillerRed-expressing neurons innervating the habenula of KR11 fish. Positive VGlut1/2 (Figure 7F) and negative GABA antibody labels (Figure 7G) were found in the habenula afferents expressing KillerRed, providing more evidence that the cluster includes homologs of the posterior septal nuclei. Altogether, these results imply that at least a subset of neurons expressing KillerRed is part of the excitatory septal-habenular pathway, representing an evolutionarily conserved projection.

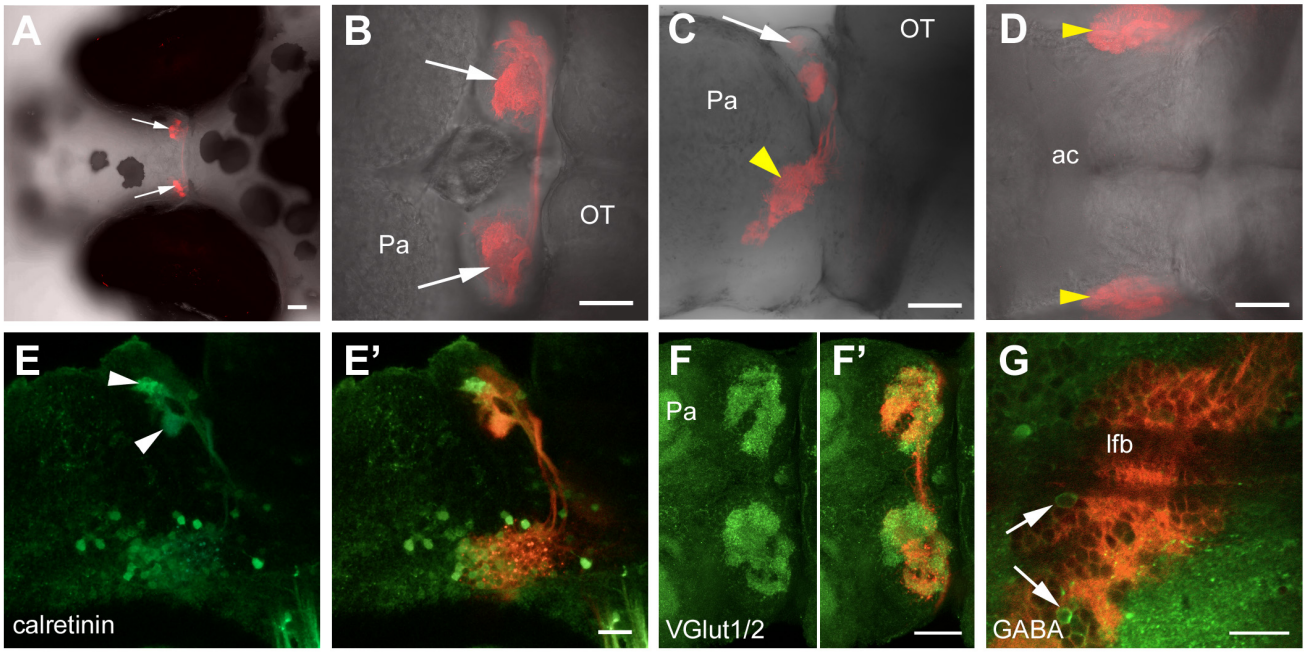

Figure 7. Expression and characterization of KillerRed in habenula input neurons. A: Dorsal view of the brain of a KR11 zebrafish at 30 dpf. KillerRed is expressed in the membrane of neurons that innervate the habenula (white arrows), a paired structure in the epithalamus. B: Dorsal view of the habenula (white arrows) at higher magnification. C: Lateral view of the same brain, showing fiber projections of the afferents into the dorsal habenula (white arrow). Cell bodies of KillerRed-expressing neurons (yellow arrowhead) are in the ventral forebrain. D: Ventral view of the same brain, showing the lateral position of the KillerRed-expressing neurons (yellow arrowheads) in the forebrain. E: Lateral view, showing calretinin label (green) in habenula afferents projecting to habenula neuropils (white arrowheads) of a 30 dpf fish; E' overlay with KillerRed fluorescence. F: Dorsal view, showing VGlut1/2 label (green) in habenula afferents; F' overlay with KillerRed fluorescence. G: Lateral view at high magnification, showing GABA label (green) and cell bodies of habenula afferents expressing KillerRed. Arrows indicate rare GABA-positive neurons in the cluster. The lateral forebrain bundle is visible in this optical section, passing through the KillerRed cluster. ac: anterior commissure; lfb: lateral forebrain bundle; OT: optic tectum; Pa: pallium. Anterior is to the left in all images. Scale bar = 50 µm for panels A-D, 20 µm for others.

In the KR4 fish, KillerRed was expressed in cells of the circumventricular organ and parapineal organ (Figure 8). The circumventricular organ does not send or receive connections to or from the habenula, while the parapineal organ preferentially innervates the left habenula. As the KillerRed-expressing cells were in close

proximity to the habenula nuclei, the region of irradiation was similar in both KR4 and KR11 zebrafish.

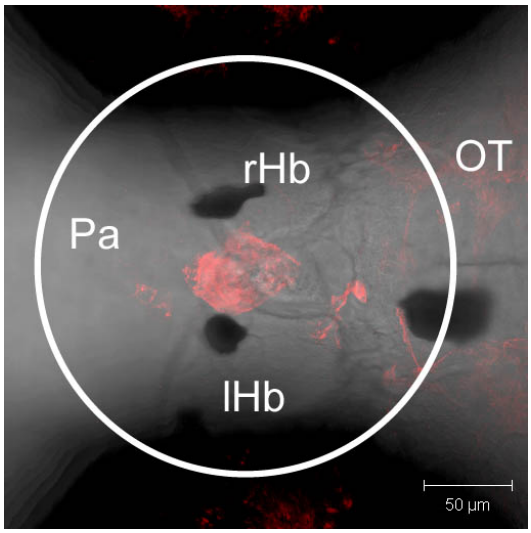

Figure 8. Expression of KillerRed in cells of the circumventricular organ and parapineal organ. Dorsal view of a KR4 zebrafish, showing KillerRed fluorescence in cells slightly anterior to the habenula. The white circle marks the region of irradiation. OT: optic tectum; Pa: pallium; rHb: right habenula; lHb: left habenula. Anterior is to the left.

Annexin V labeling. Upon irradiation with green light, KillerRed was photobleached, resulting in a loss of fluorescence (Figure 9A-B). No recovery of fluorescence was detected at three hours post-irradiation, when the fish were fear conditioned. However, fluorescence appeared dimly in axons innervating the habenula after 24 hours (Figure 9C), gradually recovering over days. Positive labeling with Annexin V demonstrated damage to the cell membrane ensuing from photobleaching. Three hours after photobleaching of the left habenula, Annexin V bound only to left habenula afferents and not efferents (Figure 9D-E). Some label was visible on axons that passed through the habenular commissure to terminate in the contralateral habenula, but no label was observed on axons that originated from the non-irradiated right side. KillerRed fluorescence remained undetected in the irradiated

left habenula. Annexin V labeling occurred within minutes of irradiation (Figure 9F), persisted for at least six hours, and was restricted to KillerRed-expressing cells that were either unilaterally or bilaterally photobleached.

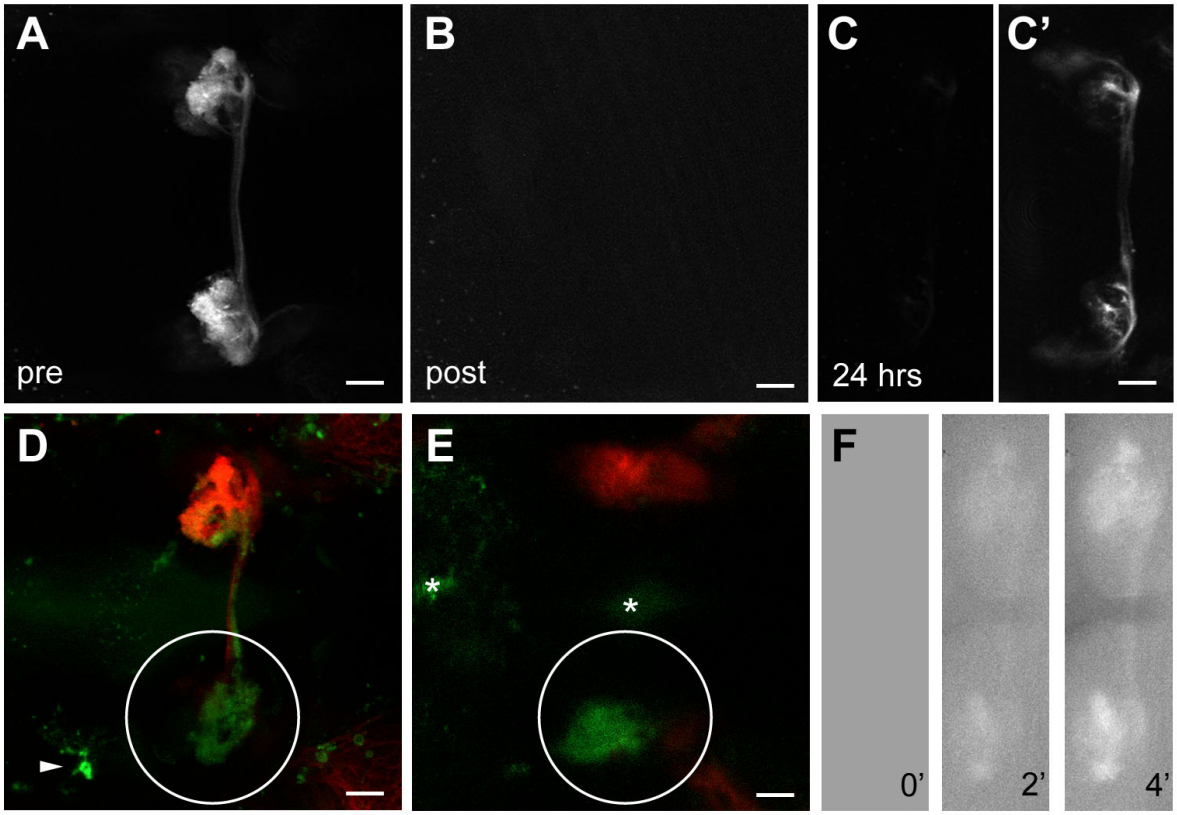

Figure 9. Bilateral photobleaching of KillerRed in KR11 zebrafish, comparing fluorescence before (A), immediately after (B), and 24 hours after (C) irradiation. A second image at 24 hours post-irradiation (C') was taken with a larger pinhole (2 airy units) on the confocal microscope to visualize dim recovery of fluorescence that was minimally detected with the settings in earlier images. FITC-Annexin V label in KR11 fish 3 hours after unilateral photobleaching of the left habenula (D). The white circle marks the region of irradiation. One cell (arrowhead), presumably undergoing apoptosis, is labeled outside the irradiated region. Deeper focus of the cell bodies in the same larva (E), showing FITC-Annexin V label in the side that was irradiated. Asterisks indicate sites of FITC-Annexin V injection. Dynamic labeling with Annexin V occurs within minutes (F), at the time when irradiation is carried out. All images are dorsal views, with anterior to the left. Scale bar = 20 μm.

Fear behavior. When KR11 fish with photobleached habenula afferents were subjected to escapable paired (ESP) conditioning, they failed to execute avoidance

responses in the probe trial (Figure 10; Movie 1). Photobleaching of KillerRed expressed in cells close to the habenula in KR4 fish did not produce this deficit in avoidance (Movie 2), which rules out the possibility that the behavior was caused by non-specific effects of photobleaching, such as damage spreading to other regions in the vicinity. Moreover, photodamaged neurons in the parapineal organ did not affect the avoidance response, regardless of whether they innervate the left habenula. Compared to irradiated KR4 controls, significantly fewer irradiated KR11 fish crossed the midline of the tank away from the LED during CS presentation (Pearson χ^2 (1, N=20) = 7.5; p = .006; Cramer's Φ = .612). Instead, irradiated KR11 fish displayed reduced mobility until CS offset, in contrast to irradiated KR4 controls (F (1, 17) = 20.522; p < .001; partial η^2 = .547).

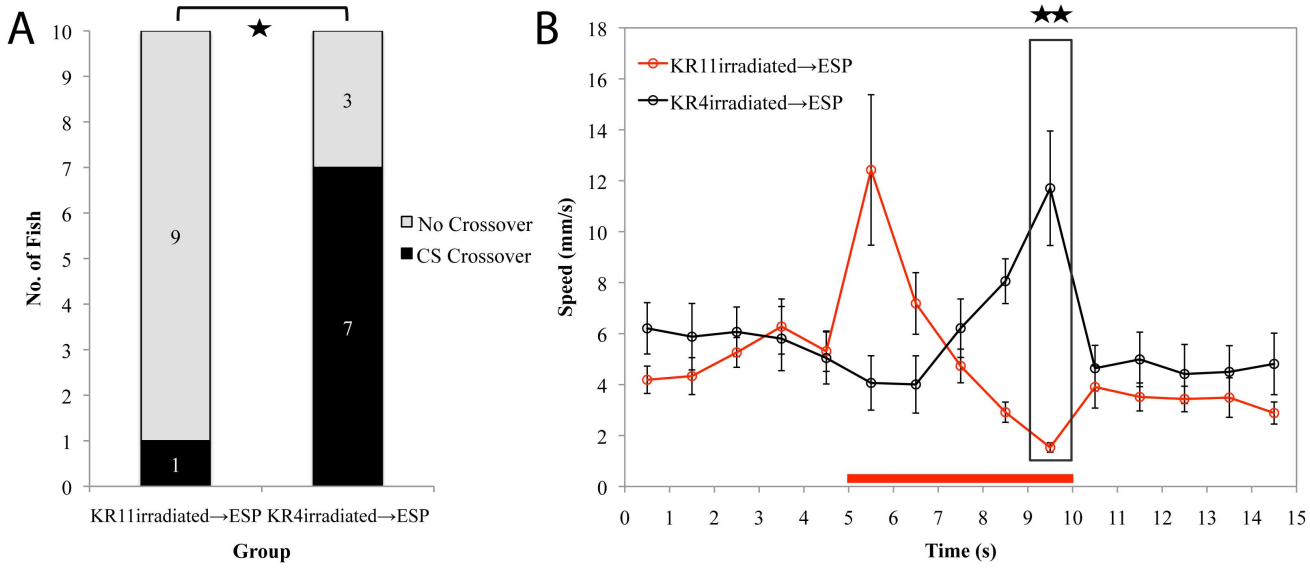

Figure 10. Midline crossover performance (A) and swimming speed (B) of the irradiated KR11 and KR4 groups in the probe trial. The red bar indicates CS presentation, and the box indicates the time point during CS presentation for which the ANCOVA analysis was conducted on swimming speeds between groups, using pre-CS speed as the covariate. Error bars indicate s.e.m., ★★ p < .001; ★ p < .05.

When KR11 fish were irradiated after the training session, they still displayed the avoidance responses in the probe trial, despite photobleached habenula afferents (Figure 11). Unlike fish irradiated before ESP training, post-training irradiation did not produce reduced mobility to the CS (F (1, 17) = 20.706; p < .001; partial η^2 = .563). Significantly more post-training irradiated fish crossed the midline before light offset, in comparison to pre-training irradiated fish (Pearson χ^2 (1, N=20) = 12.8; p < .001; Cramer's Φ = .800). These results suggest that disruption of the habenula afferents prevented the acquisition, rather than expression, of the avoidance response, since photobleaching did not immediately bias the fish towards a freezing-like response.

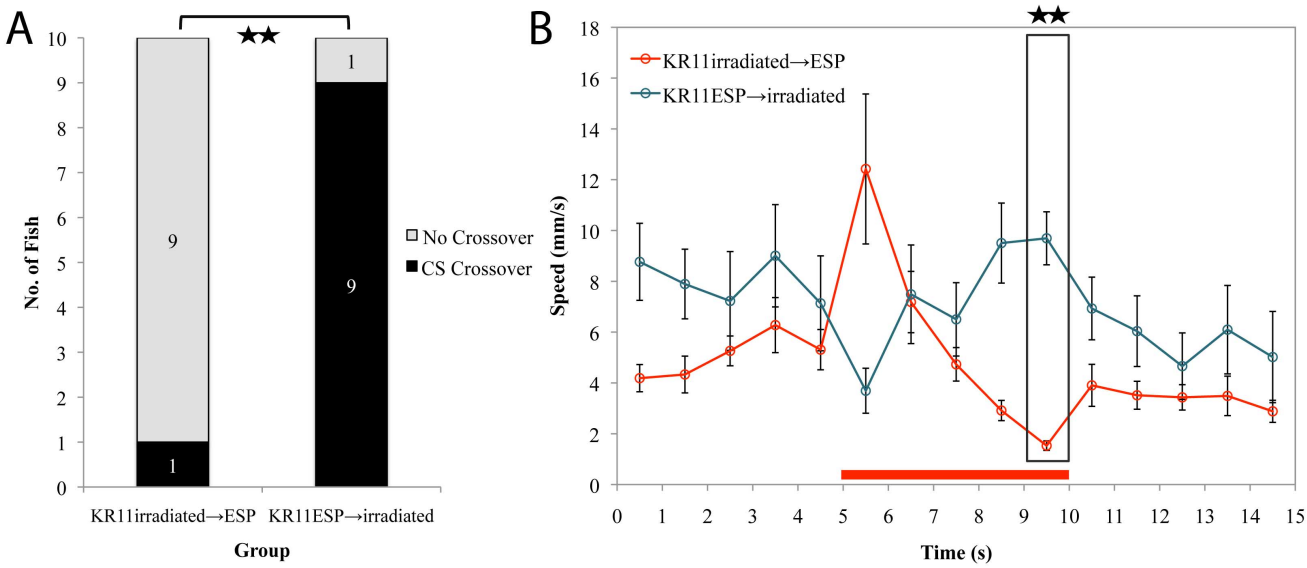

Figure 11. Midline crossover performance (A) and swimming speed (B) of the pre-training and post-training irradiated KR11 groups in the probe trial. The red bar indicates CS presentation, and the box indicates the time point during CS presentation for which the ANCOVA analysis was conducted on swimming speeds between groups, using pre-CS speed as the covariate. Error bars indicate s.e.m., ★★ p < .001.

In support of this finding, the trend of crossovers across training trials (Figure 12) showed pre-training irradiated KR11 fish displaying avoidance early in

conditioning, but fewer fish crossed the midline prior to shock delivery as the session progressed. Fish without photobleached neurons, on the other hand, successfully acquired the instrumental response; irradiated KR4 and post-training irradiated KR11 fish were both more likely to crossover as training progressed. On one of the early training trials (trial 2), one of the pre-training irradiated KR11 fish scored a crossover during an initial jolt of movement resembling a startle when the CS was presented. As this crossover was dissimilar from the other avoidance responses, we excluded it from the crossover analyses.

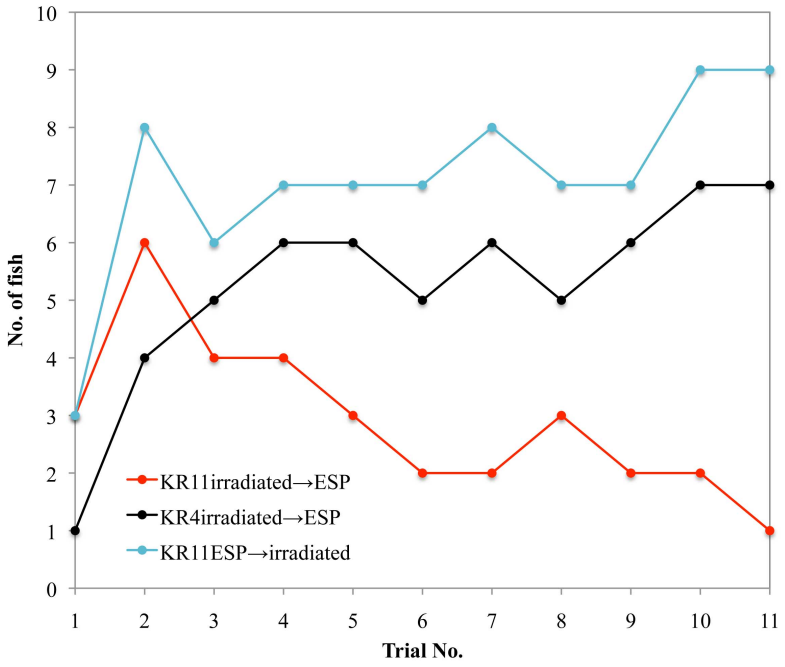

Figure 12. Number of fish, out of 10, that crossed the midline during CS presentation for each of the 10 training trials and the probe trial (Trial 11, shock not presented).

Interestingly, photobleaching of habenula afferents not only interfered with instrumental learning, but also affected the fish's behavior towards unpaired CS and US events. When trained on the unpaired procedure, irradiated KR11 fish displayed reduced mobility during CS presentation, similar to irradiated fish in the ESP

procedure (Figure 13A). This behavior differed from non-irradiated fish, which showed no particular response to the CS after unpaired conditioning in Experiment 1. An ANCOVA conducted on swimming speeds in the three conditions indicated a significant group effect (F (2, 26) = 7.395; p = .003; partial η^2 = .363), and pairwise comparisons showed statistical differences between the irradiated Unpaired group and the non-irradiated unpaired group (p = .001 (α_{pc} = .017)), whereas the irradiated Unpaired and irradiated ESP groups were not significantly different (p = .48 (α_{pc} = .05)).

The immobility was not a reaction to light or shock *per se*, as irradiated fish trained on either the CS alone or the US alone procedures did not exhibit the freezing-like response (Figure 13B). Comparing both groups with the irradiated ESP group, the ANCOVA revealed a significant group effect (F (2, 26) = 9.272; p = .001; partial η^2 = .416), and pairwise comparisons showed significantly lower speeds in the irradiated ESP group than the irradiated CS alone group (p = .013 (α_{pc} = .025)) and the irradiated US alone group (p < .001 (α_{pc} = .017)). The irradiated CS alone and US alone groups were not significantly different (p = .116 (α_{pc} = .05)).

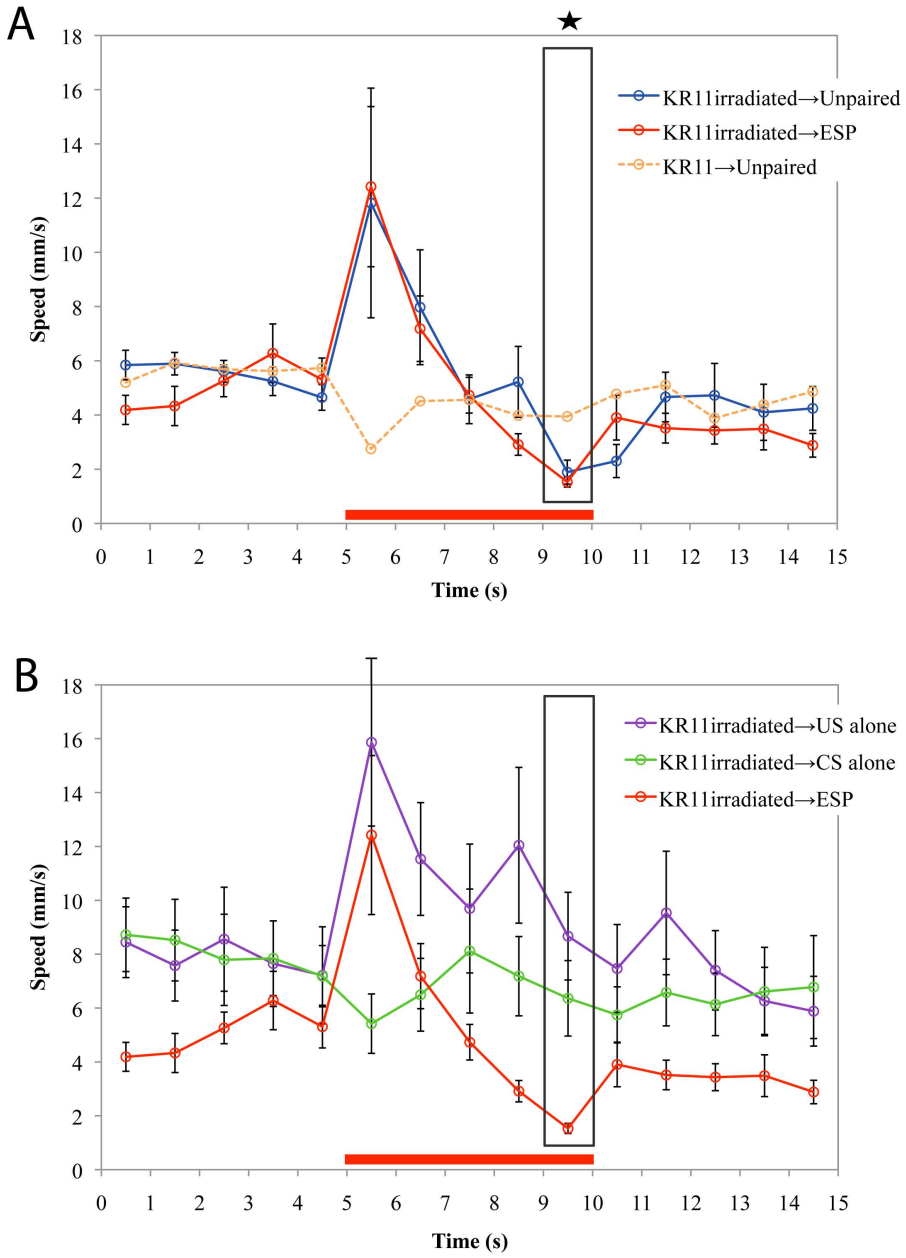

Figure 13. Swimming speeds of the irradiated KR11 groups in the probe trial. A: Contrasting irradiated unpaired controls with irradiated ESP fish and non-irradiated unpaired controls. Dotted line indicates results earlier presented in Experiment 1. B: Contrasting irradiated CS alone and US alone controls with irradiated ESP fish. The red bar indicates CS presentation, and the box indicates the time point during CS presentation for which the ANCOVA analysis was conducted on swimming speeds between groups, using pre-CS speed as the covariate. Error bars indicate s.e.m., ★ $p <$ .05.

In addition, many KR11 fish that were irradiated and subjected to electric shock displayed a startle response immediately following the onset of light in the probe trial (Figure 14A). Startle was less often observed in non-irradiated fish subject to the same conditioning procedures, and never exhibited in fish trained with light alone, i.e., they never received a shock. A two-way Chi-square test indicated significant group differences in startle during the first second of CS presentation (Pearson χ^2 (7, N=80) = 28.8; $p < .001$; Cramer's V =.60). Follow-up pairwise comparisons found marginally significant differences between the irradiated and non-irradiated ESP groups ($\chi^2 = 5.051$; $p = .025$ ($\alpha_{pc} = .025$)), unpaired groups ($\chi^2 = 3.81$; $p = .05$ ($\alpha_{pc} = .05$)), and US alone groups (($\chi^2 = 6.667$; $p = .01$ ($\alpha_{pc} = .017$)), while the CS alone groups showed no startle.

To further illustrate this relationship, the ESP, Unpaired and US alone groups were pooled, and a two-way contingency table analysis was conducted to evaluate differences in startle when shock was applied to irradiated or non-irradiated fish (Figure 14B). A significant relationship between irradiation and startle was found (Pearson χ^2 (1, N=60) = 14.7; $p < .001$; Cramer's $\phi = .495$); the probability of a fish displaying startle in response to light was about 5.67 times higher when the fish had been irradiated. Given that increased startle indicates heightened anxiety and stress (Davis et al., 2010), these results suggest that irradiated KR11 fish developed elevated levels of fear and anxiety when subjected to shock during training.

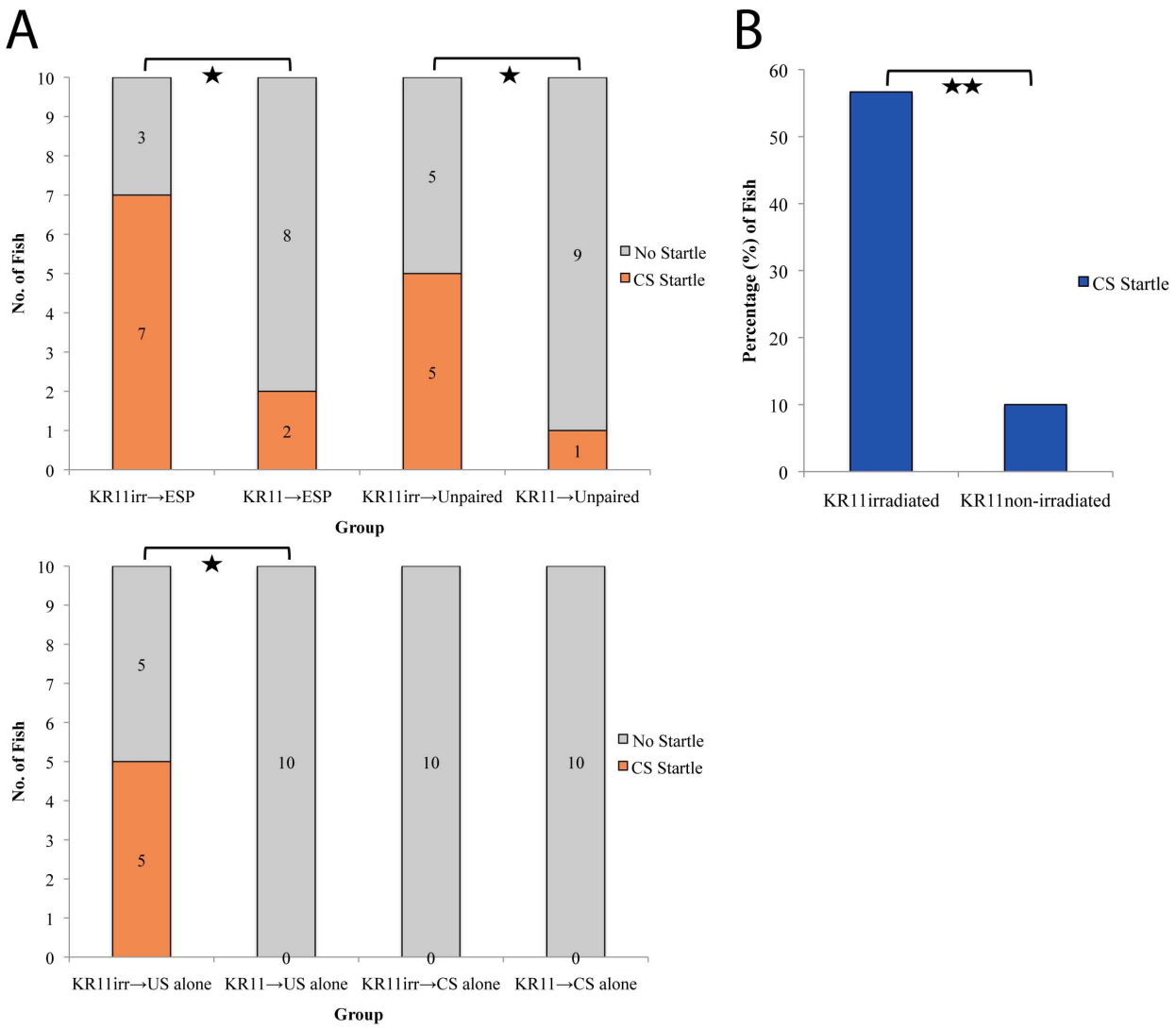

Figure 14. Startle responses of the irradiated and non-irradiated KR11 groups in the probe trial. A: Contrasting individual ESP, Unpaired, US alone and CS alone groups. B: Contrasting pooled irradiated and non-irradiated groups. ★★ $p < .001$; ★ $p < .05$.

Experiment 3

In this experiment, learned fear responses were tested following disruption of the neural pathway sending outputs from the habenula. These neurons were targeted with the light chain of tetanus toxin (TeTxLC) as a different method of manipulation. TeTxLC is a genetically encoded probe that cleaves synaptobrevin (Link et al., 1992), thus prohibiting synaptic extocytosis without killing the cells. In effect, this silences

the neurons by specifically blocking neurotransmission, but leaves the circuit otherwise intact (Baier & Scott, 2009).

Method

Generation of transgenic zebrafish lines. The GAL4^{s1019t} enhancer trap line (Baier & Scott, 2009) driving UAS:Kaede expression primarily in habenula efferents were crossed to fish carrying UAS:TeTxLC-CFP, to target expression of tetanus toxin light chain in output neurons of the habenula.

The GAL4^{s1019t}/UAS:Kaede line was obtained from the Oregon Stock Center. The GAL4/UAS system, comprising the Gal4 transcription factor and its DNA binding site, called the Upstream Activating Sequence (UAS), is a bipartite transgenic technique commonly used in *Drosophila* research (Brand & Perrimon, 1993). In cells where the Gal4 gene is expressed, the Gal4 protein targets UAS and drives expression of the downstream open reading frame (Baier & Scott, 2009). In principle, this allows genetically encoded probes linked to the UAS promoter, such as photoconvertible fluorescent protein Kaede in UAS:Kaede, to be expressed in patterns of cells expressing Gal4, like GAL4^{s1019t}. Here, Kaede serves to visualize the specific GAL4 expression pattern of the fish line; it initially fluoresces in green, but converts into red on exposure to UV light. The UAS:TeTxLC-CFP line was kindly provided by Koichi Kawakami.

The GAL4^{s1019t}/UAS:Kaede/UAS:TeTxLC-CFP triple transgenic fish were subjected to ESP conditioning, and then tested for presence of TeTxLC using antibody labeling of the CFP tagged to the tetanus toxin protein. Animals with detected TeTxLC-CFP expression were sorted into the manipulation group (n=10), while the remaining fish with undetected expression served as GAL4^{s1019t} controls

(n=10). UAS:TeTxLC-CFP single transgenic fish (n=10) were also trained on the ESP, as a second control group accounting for any behavioral artifacts of the different transgenic backgrounds.

Immunofluorescence. To verify the neurons in which TeTXlc was expressed, the larvae were labeled with a GFP antibody that recognizes the CFP tag. After fear conditioning, brains were dissected out and fixed in 4% PFA in PBS. Green Kaede fluorescent expression was photoconverted to red by irradiating the fixed tissue using the DAPI filter set on a compound microscope (Leica DM LFS), with a 10x objective, for two minutes. A solution of PBS with 1% bovine serum albumin (Fraction V; Sigma), 1% DMSO and 0.1% Triton X-100 was used to permeabilize the tissue and to dilute the GFP (Torrey Pines TP-401; 1:1000) primary antibody. The Alexa 488 goat anti-rabbit (Molecular Probes; 1:500) secondary antibody was diluted in PBS. After permeabilization, brains were incubated in the primary antibody for 12 hours at 4°C, rinsed three times in PBS and then incubated in the secondary antibody for two hours at room temperature. After three further rinses, brains were mounted in 1.2% low-melting agarose (in PBS), and imaged with a laser scanning confocal microscope (Zeiss LSM 510), using 20x, 40x and 63x water immersion objectives.

Results and Discussion

GAL4^{s1019t}/UAS:Kaede/UAS:TeTxLC-CFP expression. Expression of the GAL4^{s1019t} driver in habenula efferents was confirmed by imaging the UAS:Kaede fluorescence pattern in fish. Kaede expression was strong in the habenula, with low-level scattering in the rest of the fish (Figure 15A). GAL4^{s1019t} drives expression mainly in the dorsal (mammalian medial) habenula, with some expression in the ventral (mammalian lateral) habenula on the right side (Figure 15B). The output

neurons project primarily to the interpeduncular nucleus, and some to the raphe (Figure 15C).

While Kaede was expressed in neurons extending dendrites into more lateral regions of the dorsal habenula, TeTxLC-CFP was detected in neurons that extended dendrites into the medial neuropil of the dorsal habenula (Figure 15D). Differential expression patterns of the proteins is not surprising, due to the well-known variegation of transgenes in zebrafish (Halpern et al., 2008). A few neurons expressing TeTxLC-CFP were detected elsewhere in the brain in fish examined after conditioning, but these differed from fish to fish (Figure 15E-F). Importantly, consistent expression was observed only in the habenula.

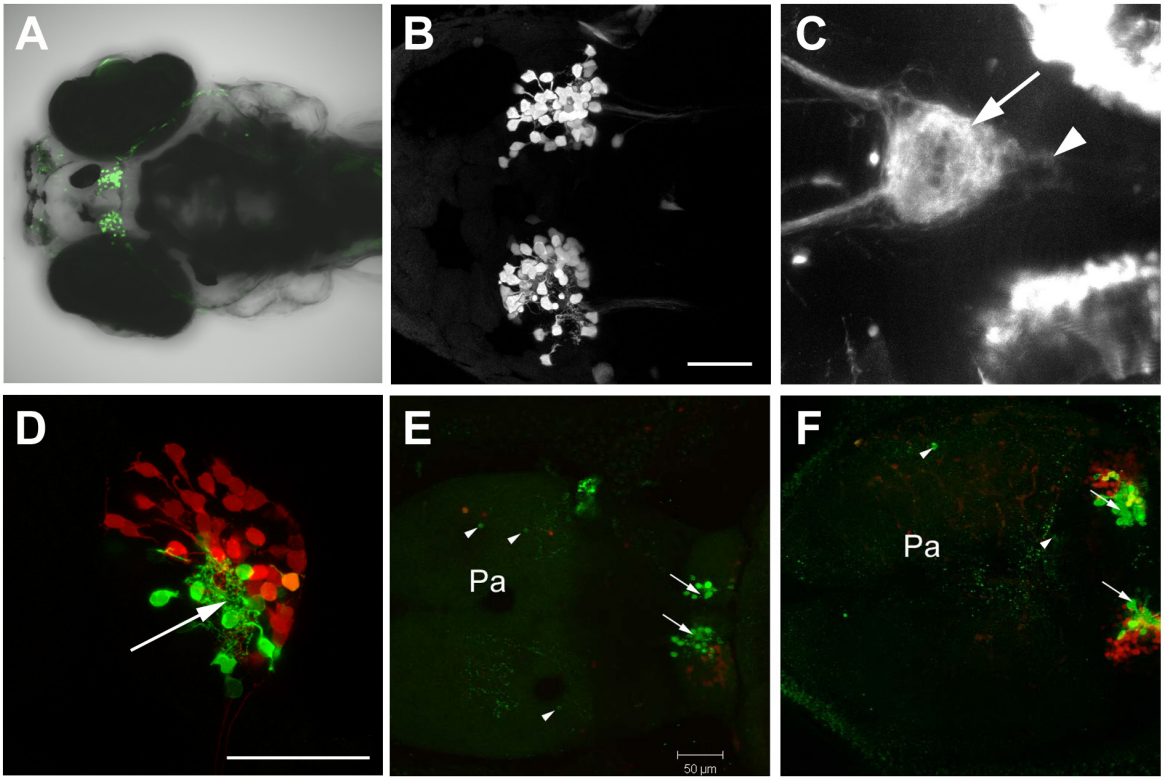

Figure 15. Expression of Kaede and TeTxLC-CFP in habenula output neurons. A: Green fluorescence of Kaede in the habenula of GAL4^{s1019t}/UAS:Kaede zebrafish. B: Higher magnification of the Kaede expression in the habenula outputs. C: Kaede fluorescence in projections of the output neurons innervating the interpeduncular nucleus (arrow) and the raphe (arrowhead). D: Red fluorescence of converted Kaede and green fluorescent labeling of TeTxLC-CFP in the right habenula of GAL4^{s1019t}/UAS:Kaede/UAS:TeTxLC zebrafish, with lateral at the top. Efferents expressing TeTxLC extend projections of dendrites into a single neuropil (arrow). E: TeTxLC-CFP (green; arrowheads) and Kaede (red) observed in several forebrain neurons in one fish. F: TeTxLC-CFP (green; arrowheads) visible in two forebrain neurons in another fish, while Kaede (red) was expressed in pericytes that occur about blood vessels and contribute to vasculature. Expression of TeTxLC-CFP was found in the medial region of the dorsal habenula of both fish (arrows). All images are dorsal views, with anterior to the left. Pa: pallium. Scale bar = 50 µm.

Fear behavior. As shown in Figure 16, fish with positively labeled TeTxLC-CFP did not display avoidance responses in the probe trial (Pearson χ^2 (2, N=30) = 17.143; $p < .001$; Cramer's V = .756). In contrast to GAL4^{s1019t} siblings that did not express TeTXlc (($\chi^2 = 13.333$; $p < .001$ ($\alpha_{pc} = .025$)) or UAS:TeTxLC-CFP fish that did not carry the GAL4^{s1019t} (($\chi^2 = 13.333$; $p < .001$ ($\alpha_{pc} = .05$)), significantly fewer

GAL4^{s1019t}/UAS:TeTxLC-CFP fish crossed the midline before CS offset. They also displayed reduced mobility, whereas control groups did not (F (2, 26) = 5.019; p = .014; partial η^2 = .279). Follow-up tests to the ANCOVA revealed significant differences between the swimming speed of GAL4^{s1019t}/UAS:TeTxLC-CFP fish and GAL4^{s1019t} controls (p = .007 (α_{pc} = .017)), and between GAL4^{s1019t}/UAS:TeTxLC-CFP fish and UAS:TeTxLC-CFP controls (p = .019 (α_{pc} = .025)). The speeds of the two control groups did not significantly differ (p = .737 (α_{pc} = .05)).

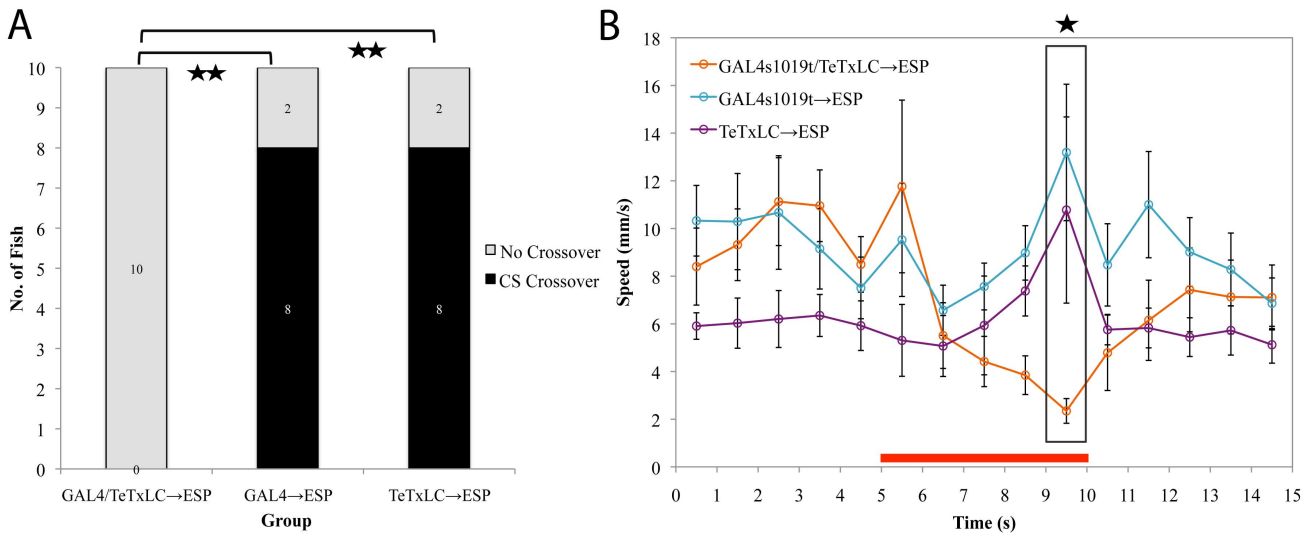

Figure 16. Midline crossover performance (A) and swimming speed (B) of the GAL4^{s1019t}/UAS:TeTxLC, GAL4^{s1019t}, and UAS:TeTxLC groups in the probe trial. The red bar indicates CS presentation, and the box indicates the time point during CS presentation for which the ANCOVA analysis was conducted on swimming speeds between groups, using pre-CS speed as the covariate. Error bars indicate s.e.m., ★★ p < .001; ★ p < .05.

Examining the groups' trend in avoidance across the conditioning session, GAL4^{s1019t}/UAS:TeTxLC-CFP fish showed initial avoidance responses to cross the midline away from the CS during the first half of training, but these diminished over the remaining trials (Figure 17). The number of GAL4^{s1019t} and UAS:TeTxLC-CFP controls that exhibited an avoidance response increased as training progressed.

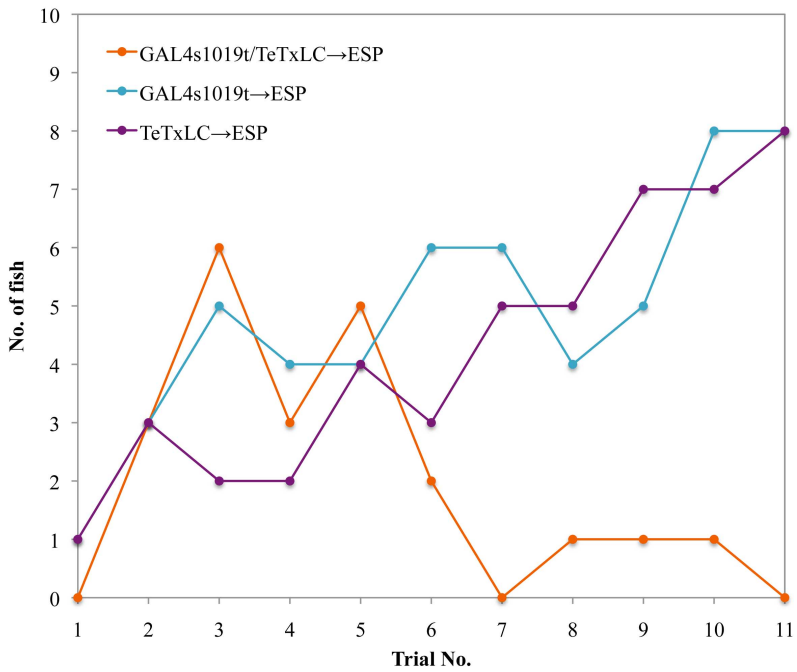

Figure 17. Number of fish, out of 10, that crossed the midline during CS presentation for each of the 10 training trials and the probe trial (Trial 11, shock not presented).

Summary and Overall Discussion

The present series of experiments provide evidence that the habenula is required to mount appropriate avoidance responses during fear learning. As shown in Experiment 1, larval zebrafish learned the contingency between CS-US pairings, and swam away from the light cue to avoid the brunt of shock delivered on one side of the tank (ESP). The observed crossovers were specific to the CS signaling oncoming escapable shock, as fish that experienced unpaired CS and US, or CS alone, did not exhibit CS crossovers. However, when shock was delivered on both sides of the tank, rendering it inescapable (ISP), fish displayed reduced movement - a freezing-like response - instead of avoidance. When fish were preexposed to inescapable shock (IS→ESP), they also displayed the freezing-like response in subsequent training, even though the shock was escapable. Disrupting specific sets of habenula afferents

(Experiment 2) and efferents (Experiment 3) both led to a switch in learned behavior from flight to a freezing-like response during ESP training, preventing fish from avoiding the shock. This change in behavior was not due to reduced sensitivity to shock, because the fish still reacted violently to the shock when it was delivered. The behavior was also not simply a result of greater exposure to shock *per se*, since irradiated fish with maximum degree of exposure to shocks in the US alone procedure did not exhibit freezing-like responses. Disruption after training did not impair avoidance during the probe trial, thus implicating the habenula in the acquisition, rather than expression of the instrumental response.

The results also demonstrate successful use of transgenic methods to target and manipulate specific neurons *in vivo*. Upon irradiation of the genetically encoded photosensitizer KillerRed, Annexin V bound to photobleached neurons, indicating lipid peroxidation resulting from oxidative stress in cells, which affects membrane proteins and impairs electrophysiological function of neurons (Balasubramanian et al., 2001; Pellmar, 1986; Pellmar & Lepinski, 1992). This confirms the expected damage by superoxides released during irradiation of KillerRed. Photodisruption of input neurons to the habenula was performed with a high degree of temporal and spatial resolution beyond that achieved by invasive lesion techniques. For instance, the lateral forebrain bundle passing through the cluster of afferents did not express KillerRed (Figure 7G; Movie 3), and hence was not subject to manipulation by the procedure. Despite being close to the KillerRed-expressing cells, it is unlikely that cells of the lateral forebrain bundle were extraneously photodamaged in the process, since successful avoidance by irradiated KR4 fish indicates that the damage is relatively specific to regions targeted with KillerRed and does not spread to regions in the vicinity. Although this optogenetic approach has previously been shown to cause

cell fragmentation and death *in vitro* (Bulina et al., 2006a; 2006b), these results extend the use of KillerRed's phototoxicity to damage neurons and impact behavior *in vivo*.

Expression of tetanus toxin light chain to inhibit neuronal activity is a well-established method used in zebrafish (Asakawa et al., 2008; Koide et al., 2009), as well as *Drosophila* (Sweeney et al., 1995) and mice (Yamamoto et al., 2003). Specific expression of TeTxLC in efferents of the zebrafish dorsal (homologous to mammalian medial) habenula also led to avoidance defects, similar to that found in fish with photobleached KillerRed-expressing afferents. Results from this second perturbation technique not only provide support for the behavioral consequences of KillerRed manipulation, but also imply that silencing the zebrafish dorsal (mammalian medial) habenula outputs are sufficient to cause the switch in defensive behavior from flight to freezing-like responses. The TeTxLC-CFP-positive neurons extended dendrites into the medial neuropil of the dorsal habenula, the same region in which calretinin-positive neurons marking the BNSM were detected. In addition to calretinin, the KillerRed-expressing cluster of afferents showed overlapping expression with VGlut1/2 antibody labels marking glutamergic synapses, but not with GABA antibody labels, similar to that reported in the nucleus septofimbrialis (SFi) and the nucleus triangularis (TS) projecting to the mammalian medial habenula (Qin & Luo, 2009). The SFi and TS cells contain both calretinin and calbindin, with 100% co-localization in rats (Sperlágh et al., 1998). Identical distributions of calretinin and calbindin antibody labels have been found in the KillerRed-expressing cluster of KR11 zebrafish (S. Jesuthasan, personal communication, August 4, 2010). Taken together, it appears that the inputs from the posterior septum to the zebrafish dorsal (mammalian medial) habenula are critical for avoidance learning. When disrupted,

larval zebrafish behave as if subjected to inescapable shock, passively tolerating rather than avoiding the aversive outcome even when it is escapable. These findings are in line with Wilcox et al.'s (1986) suggestion that impaired avoidance performance arises from disruption of the septal-medial habenula-interpeduncular nucleus pathway.

Importantly, disruptions did not result in immediate behavioral deficits. Rather, the helpless behavior developed over the course of training, after the fish encountered repeated trials of shocks. This was also the case for fish exposed to inescapable shocks prior to escapable training. Lack of avoidance during training, in turn, set the fish up for harsher shock conditions. Since repeated shocks aggravate the stressfulness of the experience, stress would build up over the training session, like it does during a preexposure to inescapable shock. Indeed, stress can elicit freezing in response to a stimulus that would normally trigger flight (Mongeau et al., 2003).

The loss of avoidance responses over training following KillerRed or TeTxLC manipulations is consistent with previous reports that habenula lesions only affect learning behavior in stressful situations; avoidance was impaired only when the severity of shock was increased, or when physical effort required for avoidance was increased (Thornton & Bradbury, 1989). In addition, a recent study revealed that habenula-lesioned mice that were previously stressed by fear conditioning showed impaired pre-pulse inhibition, hypolocomotion in an open field, and greater hyperlocomotion in response to the dopamine agonist apomorphine, compared to controls (Heldt & Ressler, 2006). Considering the habenula's connectivity, the fact that disruptions to the habenula induce learning deficits that are exacerbated by stress, and enhanced sensitivity to dopamine agonists, suggests that the habenula plays an active role in modifying monoamine transmission and consequently regulates

monoamine-dependent behaviors subsequent to aversive events. Without a functional habenula, the animal lacks behavioral flexibility and/or feedback to cope with the threatening situation appropriately, and ultimately spirals into a helpless state. In other words, as Lecourtier & Kelly (2007) described, damage to the habenula induces a "hypersensitivity to stress" (p.659), such that the organism becomes impaired at adapting to stress, and especially susceptible to the 'learned helplessness' behavioral sequelae activated by uncontrollable stressors. Thus, habenula activity appears to modulate the stressful impact of an event and influence coping strategies that control the outcome of the experience.

How, then, does the habenula interact with stress circuitry and contribute to successful avoidance learning? One hypothesis is that the habenula signals a control component in the network to orchestrate appropriate avoidance responses.

According to a series of experiments conducted in Steven Maier's laboratory, uncontrollable stress selectively activates and sensitizes serotonergic (5-HT) neurons in the DRN to produce the 'learned helplessness' behavior. More recently, they found that control over the aversive experience inhibits this stress-driven activity, thereby enabling more active coping behaviors. Amat et al. (2005) proposed that a neural circuit involving the ventral medial prefrontal cortex (vmPFC) processes whether a stressor is, or is not, controllable and then regulates DRN function accordingly. Glutamatergic projections from regions within the vmPFC synapse onto predominantly GABAergic neurons in the DRN, which in turn inhibit 5-HT neurons (Jankowsi & Sesack, 2004). When the $GABA_A$ receptor agonist muscimol was microinjected into the vmPFC to inhibit its activity, rats exposed to escapable shock showed significantly increased 5-HT neuronal activity in the DRN that was comparable with rats given inescapable shock (Amat et al., 2005). The muscimol led

to poor escape responding and increased freezing in escapable-shocked rats, similar to behaviors displayed by inescapable-shocked rats. In contrast, rats microinjected with vehicle showed the expected low levels of 5-HT activity after the escapable stressor, regular avoidance behavior, and minimal freezing. From these results, the authors concluded that the presence of control drives the vmPFC to inhibit serotonergic activity in the DRN and prevent the cascade of events leading to learned helplessness. Of note, there is also the possibility that the vmPFC may regulate the DRN indirectly through projections to other structures, which in turn regulate the DRN (Amat et al., 2005). Interestingly, Varga, Kocsis, and Sharp (2003) have reported a convergence of medial PFC and lateral habenula outputs onto the same non-serotonergic, presumably GABAergic, neurons in the DRN. Moreover, experiments showed that habenula lesions affect 5-HT release and learned helplessness behavior (Amat et al., 2001).

It is plausible that the septal-habenula pathway is part of the circuit that evaluates contingency between the organism's behavior and outcome, and signals a measure of control over a stressor. In mammals, the lateral habenula neurons project to midbrain areas involved in the release of serotonin (the dorsal and median raphe nuclei) and dopamine (the substantia nigra pars compacta and ventral tegmental area), while the medial habenula neurons project to the interpeduncular nucleus, which projects to the raphe nuclei and other areas (Hikosaka, 2010). Through regulatory neural connections, the habenula may influence serotonergic signals that monitor the stressfulness of a situation, and dopaminergic signals that serve as reward prediction errors to shape effective behavioral strategies. In this scenario, positive feedback denotes presence of control, while negative feedback represents lack of control. To speculate further, the present findings suggest that the mammalian medial (zebrafish dorsal) habenula, similar to the vmPFC, signals the presence of control, whereas, the

mammalian lateral (zebrafish ventral) habenula signals the lack of control, given its activation by negative reward (Matsumoto & Hikosaka, 2007).

As controllable stress produces less conditioned fear and anxiety than does uncontrollable stress (Maier & Watkins, 1998), it follows that the mammalian medial (zebrafish dorsal) habenula also functions to downregulate anxiety following a successful response to a threat. Conversely, the absence of perceived control, i.e. absence of mammalian medial (zebrafish dorsal) habenula signaling, would intensify anxiety. Startle is an indicator of anxiety (Davis et al., 2010), and in Experiment 2 of the current study irradiated KR11 zebrafish subjected to shock demonstrated significantly increased startle responses, implying elevated levels of anxiety in these fish compared to non-irradiated fish subjected to equivalent shock. This finding parallels the larger potentiation of startle during fear conditioning experiments conducted with patients with pathological anxiety, such as panic disorder (Grillon et al., 1994)_and posttraumatic stress disorder (Grillon et al., 1998), compared to healthy controls. In a meta-analysis of 45 studies examining fear conditioning in anxiety disorder patients, Lissek et al. (2005) showed stronger overall conditioned fear responding among anxiety patients versus healthy controls during the acquisition as well as extinction of fear learning, suggesting greater “excitatory” fear conditioning and diminished “inhibitory” fear extinction in anxiety patients. Included in the studies, Grillon and Morgan (1999) reported that PTSD patients but not healthy controls exhibited fear-potentiated startle to a CS signaling safety from the aversive US, leading to a theory that pathological anxiety arises from a failure to inhibit fear responses in the presence of safety cues. It is also possible that anxiety disorder patients experience elevated stimulus generalization in a stressful threatening context.

Interestingly, Experiment 3 did not reveal significantly greater startle in GAL4^{s1019t}/UAS:TeTxLC-CFP zebrafish despite having silenced dorsal habenula efferents and exhibiting helpless behavior. A possible explanation is that these fish experience substantially heightened anxiety as TeTxLC is permanently expressed in the dorsal habenula. According to Walker et al. (1997), startle increases with moderate levels of stress and anxiety, but diminishes at high levels. In fact, different tests of anxiety conducted with adult GAL4^{s1019t}/UAS:TeTxLC-CFP fish in our laboratory showed exaggerated alarm responses triggered by the species' alarm substance introduced to the tank, as well as prolonged durations spent in the bottom half of a novel tank, thus supporting the idea that these transgenic fish are abnormally anxious on exposure to a stressor.

Heightened anxiety may also account for the freezing-like behavior observed in irradiated KR11 fish during unpaired training. The fact that the animals showed a response to the CS suggests that some fear learning has occurred. In this case, fear responses may arise from greater contextual fear conditioning, possibly due to higher anxiety levels in the fish. Grillon and Davis (1997) previously reported such an effect in humans, showing that explicitly unpaired CS and US led to elevations in baseline startle and enhanced contextual fear-potentiated startle during a second conditioning session. The elevated baseline startle may reflect increased anxiety on re-experiencing the aversive conditioning procedure, while enhanced fear-potentiated startle demonstrates greater fear responses in the threatening context. Similarly, PTSD patients, in comparison to controls, showed generally elevated baseline startle with increased contextual fear when the experiment involved both safe and dangerous conditions (Morgan et al., 1995; Grillon et al., 1998), whereas they did not differ in

baseline startle magnitude when experimental stress was absent (Grillon et al., 1996; Grillon et al., 1998).

Moreover, the freezing-like behavior was not observed in other control fish trained with CS alone or US alone. The contrast between the unpaired control and the CS alone and US alone procedures is that unpaired light was repeatedly presented in the same training environment in which shock was delivered, so the CS could have been integrated into the context of the aversive experience and become sufficient to trigger contextual fear responses – typically freezing behavior – when presented. On the other hand, when the CS was omitted from the shock environment (US alone), the light was not perceived as part of the training context, and thus did not elicit freezing in the probe trial. This further indicates that the freezing-like behavior was not a simple potentiation of response to the CS after exposure to the US. Finally, fish that never experienced threat in the environment (CS alone) had no reason to respond in fear to repeated lights.

However, one might argue that the present findings do not necessarily provide evidence of enhanced contextual fear conditioning because the unpaired CS was still a discrete cue, and behavioral data was not collected during the intertrial intervals. As such, bouts of freezing to the training environment were not objectively measured in a time window that permits conclusions to be drawn about contextual conditioning. To verify the claim, one possible addition to the study's procedure is to measure the response to the CS in a different environment where the fish did not receive shock. However, this may not be sufficient to conclude contextual fear response to CS, as it may still be perceived as a general indication of danger and transfer fear to the new environment. Alternatively, post-training responses to a new neutral stimulus administered to the same training environment may be examined. Since this second

stimulus does not occur during the training session, it should not be integrated into the context of the aversive experience to elicit a contextual fear response. However, it is important to bear in mind that introducing a novel event to an existing threatening context may instigate a separate sensitization reaction, especially when the fish are in a state of high anxiety. Also, this method additionally requires the fish to first discriminate between the unpaired CS and the new stimulus. We previously found poor discrimination learning in the larval fish when using two light stimuli (red LED and blue LED) as CSs (Lee, 2008). Perhaps employing a different modality, such as an auditory tone, for the new stimulus would improve discrimination from the light CS. Another strategy for measuring contextual fear conditioning in the fish is to include a re-exposure to the shuttle box after training, for specific observation of fear behavior to the context alone.

Of note, the current paradigm uses colored lights and mild electric shocks to test learned fear responses, which provide the advantage of better control over the parameters of variables like duration and intensity of delivery in the conditioning procedure. While they enable more consistent training across animals, in reality the stimuli have little relevance to survival in the fish's natural environment. This may pose a limitation to comparisons with clinical and subclinical panic and phobias that generally develop toward stimuli such as heights, crowds, animals, and illness or blood injury (Agras, Sylvester, & Oliveau, 1969) that relate to potentially threatening situations recurrent in life. From an evolutionary perspective, pre-disposed aversion and precaution taken against these factors have high adaptive value across phylogeny as they promote survival. In relating fear conditioning to pathological anxiety, Lissek et al. (2005) emphasized the use of evolutionarily prepared CSs to elicit fear processes akin to those activated in human anxiety disorders. They argued that

prepared stimuli bring about faster fear learning and stronger fear responses that are more resistant to extinction and may therefore yield distinct fear conditioning results from paradigms with nonprepared stimuli. These considerations may not be particularly pertinent to the present investigation, as the fish appear to learn the relationship between stimuli quickly, displaying reliable responses by the end of a single training session. Furthermore, Grillon et al. (1998) demonstrated heightened anxiety in PTSD patients using a similar paradigm wherein participants were administered electric shocks in the presence of a light signal. Although the stressful context was not a situational reminder of their trauma, the anxiety patients still exhibited abnormal contextual fear responses, indicating that the shortcomings in their affective response system was not limited to trauma-related stimuli.

Nevertheless, it would still be meaningful to extend investigations of fear behavior in larval zebrafish using more ecologically relevant threat stimuli. The predominant environmental danger to the fish is an encounter with a predator. Apart from shock, aversive conditioning paradigms with adult zebrafish have also employed the alarm substance as the US paired with light (Hall & Suboski, 1995) or neutral odorant CSs (Suboski et al., 1990). The alarm substance is a chemical derived from injuring the skin of a conspecific; upon detection, it activates a specific pattern of antipredator fear behavior in the fish known as the alarm response (Jesuthasan & Mathuru, 2008). Applied to larval-based paradigms, the alarm substance could also serve as a useful stimulus to study the neural substrates of innate fear in the zebrafish. It would be interesting to uncover any differences in fear circuitry underlying learned versus innate fear behavior of the fish, and further pinpoint possible mechanisms of interaction between the two processes to explain why some stimuli are more effective at acquiring affective properties to activate the fear system. Such elucidation would

complement data showing that some clinically defined phobias are co-determined by genetic factors and individual experiences (Kendler et al., 1992).

Future Directions

Although the present results demonstrate that the habenula is involved in stress-related fear responses in zebrafish, the experiments did not address how this structure exerts effects on behavior. In light of the complex connections between the habenula and multiple regions in the brain, much work remains to elucidate the details and dynamics of how the neural network is organized to mediate reaction to threat. For instance, in mammals, the lateral habenula inhibits dopaminergic release in the midbrain (Christoph et al., 1986; Matsumoto & Hikosaka, 2007) via the rostral medial tegmental nucleus (Jhou et al., 2009), and inhibits serotonergic neurons in the raphe (Wang & Aghajanian, 1977) via GABAergic interneurons (Nishikawa & Scatton, 1985), but monoaminergic regulation by the medial habenula has not been reported. The medial habenula conveys information to the interpeduncular nucleus, which also projects to the raphe nuclei and the ventra tegmental area (Lecourtier & Kelly, 2007), supporting the possibility of an indirect influence on dopaminergic and serotonergic neurons (see Appendix). Further investigations of how the habenula modulates monoaminergic transmissions in the zebrafish, and how its disruption affects the neurotransmitter systems, would advance our understanding of the fear network and the basis for differentiated defensive behavior. Also, neurons upstream of the habenula afferents need to be explored in relation to external fear stimuli, internal stress responses, and interactions with the habenula and the rest of the network. It would seem necessary to uncover associations between the habenula and other well-known neural substrates of fear, such as the amygdala and the periaqueductal gray, in order to understand the fear system as a whole.

Besides the essential role of the central nucleus of the amygdala in the expression of the fear response, the basolateral amygdala has also been heavily implicated in the neural circuitry of fear. Within the basolateral complex, the lateral nucleus of the amygdala receives uni- and polymodal sensory information from cortical regions, as well as less processed subcortical input from the thalamus (Öhman & Mineka, 2001). Lesions in this region have led to deficits in the acquisition of CS-US contingencies to predict aversive outcomes in auditory fear conditioning paradigms (Campeau & Davis, 1995; Wilensky, Schafe & LeDoux, 1999), which suggests that CS and US information converge in the lateral nucleus of the amygdala to be integrated and relayed to the central nucleus and downstream for appropriate affective behaviors. While lesioning the lateral nucleus produced a loss of fear CRs including freezing (LeDoux et al., 1990), disrupting the habenula did not abolish fearful responses, indicating that fear acquisition still occurred. It is possible that the amygdalar component of the fear network interacts with the habenula in a feedback manner to dynamically shape coping behavior and regulate the level of fear and anxiety during the experience. Put simply, the amygdala ignites fear that drives the habenula to orchestrate a defensive response against threat, which in turn dampens neuronal activity in the amygdala as positive outcomes are attained and fear extinguished. The neural mechanisms of this interaction await verification.

Based on topological connectivity and histochemical expression patterns, the medial region of the dorsal telencephalic pallium of actinopterygian fish is thought to be homologous to the amygdala in mammals (Braford, 1995; Wullimann & Rink, 2002; Northcutt, 2006). In terms of function, Portavella and colleagues demonstrated a loss acquisition (Portavella et al., 2004) and retention (Portavella, Torres & Salas, 2004) of conditioned avoidance responses after lesioning the medial pallium in

goldfish, similar to results obtained with amygdalar lesions in mice. To date, the corresponding structure has been not yet been clarified in the zebrafish pallium, although existing evidence with the goldfish provide further reason to believe that the pattern of organization of the fear system is conserved across ray-finned fishes and land vertebrates. With the larval paradigm, investigations down the line can focus on the medial pallium to identify the specific set of neurons analogous to the amygdala in the forebrain of zebrafish, and characterize its connections with the habenula and other substrates of fear.

To complement the present results, one may additionally aim to rescue the impact of habenula disruption on behavior by administering pharmacological interventions to compensate for disruption-induced defects in the network. Anxiolytic drugs can be easily delivered to the zebrafish nervous system through uptake via the embryo water. One candidate for such investigations is nicotine, which produces anxiolytic effects in zebrafish (Levin, Bencan & Cerutti, 2007). It has been shown that doses of nicotine increased neuronal firing in CA3 hippocampal neurons of rats via activation of nicotinic acetylcholine receptors (Huang et al., 2010); a high density of these receptors are located in the medial habenula-interpeduncular nucleus pathway (Grady et al., 2009) and may trigger neuronal firing when exposed to appropriate concentrations of nicotine. If nicotine treatments can rescue avoidance behavior following habenula disruption, it would provide further indications of elevated anxiety levels induced by the neuronal damage. However, nicotine is also known to enhance discrimination learning in zebrafish (Levin et al., 2006), which potentially introduces a confound to any improved behavioral effects of the drug. Other commonly used anxiolytics in human and rat models, namely busipirone and diazepam, have also been found effective on zebrafish (Bencan, Sledge & Levin,

2009). These compounds act through different transmitter receptor systems; busipirone is a serotonergic ($5HT_{1A}$) receptor agonist and diazepam a benzodiazepine-GABA-A receptor agonist. Interestingly, non-addictive busipirone and diazepam have been trialed to replace the reinforcing anxiolytic effect of nicotine or relieve withdrawal symptoms in attempts to quit smoking (Hughes, Stead & Lancaster, 2010). These may serve a helpful option for attempting rescue of avoidance after disrupting the habenula.

Alternatively, one can design a reversed manipulation to stimulate the habenula and expect an opposite effect on behavior. Another method of study is to demonstrate activity of the habenula neurons in response to the fear-eliciting stimuli. If such activations were predictive of specific responses, it would provide further support for the habenula's involvement in fear behavior.

For these strategies, the zebrafish conditioning paradigm proves to be a powerful avenue of research because development of optogenetic tools in zebrafish has been on the rise. Some zebrafish transgenic lines offer the opportunity to excite specific neurons through targeted expression of the light-inducible glutamate receptor (LiGluR; Douglass et al., 2008) or the light-gated cation channel, ChannelRhodopsin (ChR2; Szebota et al., 2007); both activate neurons in response to light by opening ion channels, allowing cation influx and depolarization of affected neurons. At the same time, genetically encoded calcium indicators, such as GCaMP (Sumbre et al., 2008) and Inverse Pericam (Li et al., 2005), have been used to monitor neuronal excitatory activity in zebrafish. Hyperpolarization can also be examined using genetically encoded fluorescent sensors for chloride (Markova et al., 2008), which surely contributes to the dynamics of neural networks. With this expanded range of techniques, experiments to investigate learned fear in zebrafish are only limited by the

transgenic lines made available for study. Converging evidence from photodisruption, photoactivation, and optical imaging studies *in vivo* would strongly implicate the habenula as a critical component in the neural circuitry of fear.

Another advantage of using the zebrafish model is the fact that it is an established genetic system with significant (70-80% and higher) nucleotide and amino acid sequence homology to humans (Gerlai, 2010). Large-scale ethyl nitrosourea (ENU)-based chemical mutagenesis has discovered specific genes in the zebrafish genome (Knapik, 2000) and can be further utilized with behavioral test paradigms to screen and identify genes associated with excessive fear responses (Jesuthasan, 2011). Extending genetic predispositions to abnormal fear in humans may aid clinical diagnoses of some anxiety patients and inform us of its pathogenesis. Extensive behavioral phenotyping conducted with ENU-mutagenized mice have already revealed a number of mutant pedigrees displaying increased fear- and anxiety-related behaviors (Cook et al., 2007). The GAL4^{s1019t}/UAS:Kaede/UAS:TeTxLC-CFP transgenic zebrafish with disrupted dorsal (mammalian medial) habenula is but an example of genetically sensitizing the fear system in the animal. Combining these genetic models with pharmacological approaches, the zebrafish provides an avenue of researching new drugs for coping with fear- and anxiety-related disorders.

Conclusion

The habenula has been shown to participate in the neural network underlying fear learning and orchestration of suitable defensive responses to threat. The zebrafish brain is both genetically and optically accessible (Baier & Scott, 2009), and exhibits reliable capacity for complex behavior. With tools to silence, activate or record activity of neurons, the zebrafish model provides an effective means of analyzing

circuits and investigating cognitive phenomena in a vertebrate. As illustrated here with KillerRed, the role of specific neurons can be non-invasively tested with optogenetic manipulations in transparent larval fish.

The present study establishes that zebrafish habenula afferents from the diencephalon/forebrain are required for acquiring the appropriate conditioned fear response. When these neurons are disrupted, fish freeze instead of fleeing from escapable shock, appearing as if they lack control over the aversive outcome. This finding suggests that the habenula neurons are involved in a pathway signaling control, which mediates successful avoidance behavior and inhibits the cascade of neural events that result in helplessness. A further interpretation of this hypothesis is that the perceived ability to control or cope with a situation can buffer individuals against the negative impact of stress. Disruption of the control pathway prevents pertinent regulation of the monoaminergic system, and consequently produces dysfunctional stress responses. If so, enhancing this circuit's influence over stress-responsive neural substrates may be an important mechanism for tackling some mental disorders that involve uncontrollable anxiety and helplessness.

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Appendix

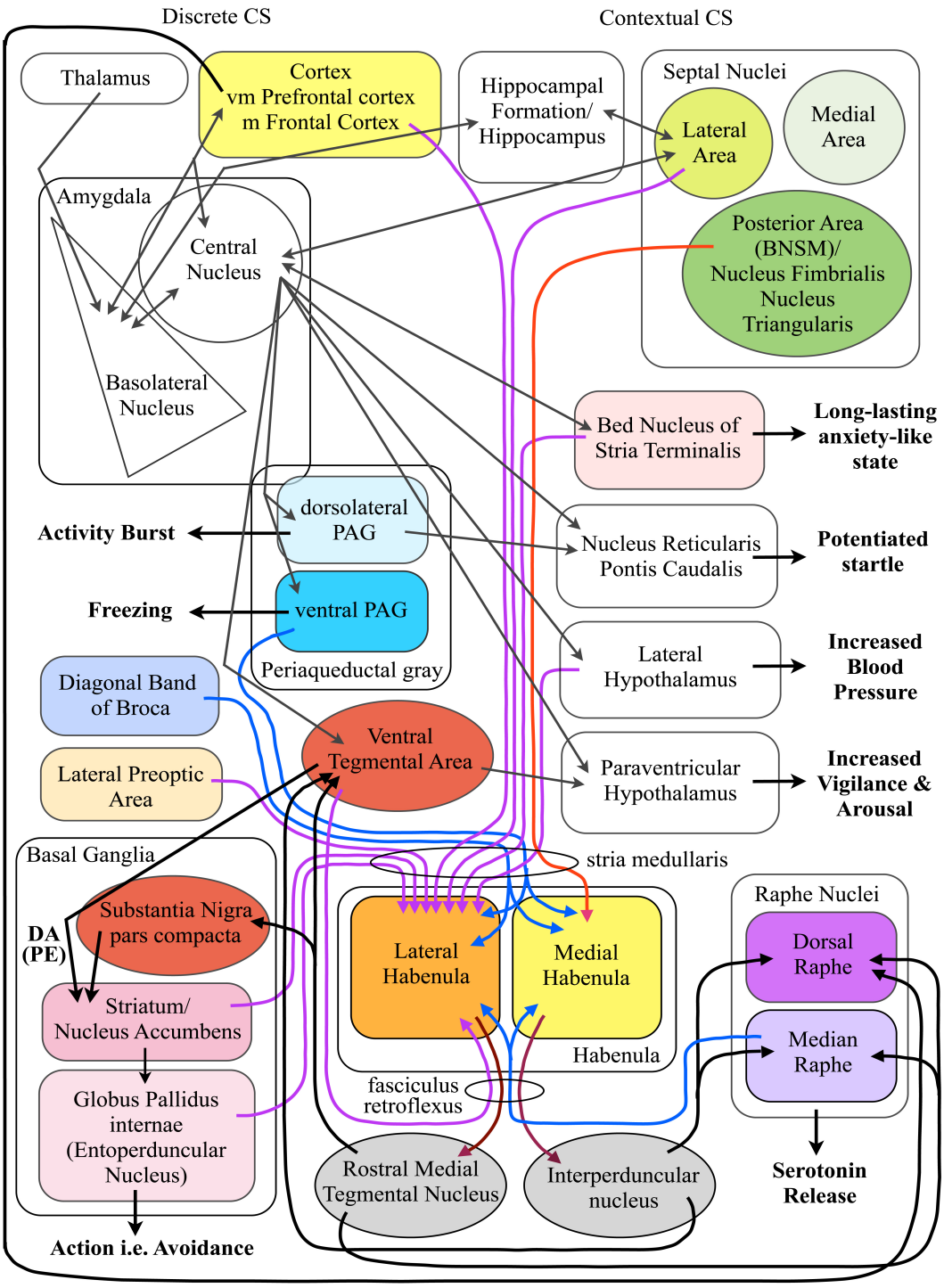

Figure 18. Hypothetical neural network of the established components of the fear circuitry (in grey) alongside connections to and from the habenula (in colour). Dopamine (DA) release sends prediction error (PE) signals to the striatum and other structures. Serotonin release is transmitted to the striatum, the substantia nigra, the hippocampus, and other brain regions.